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THE UNIVERSITY OF ALBERTA

IN VITRO MECHANISMS OF MURINE B CELL ACTIVATION

by

© RATI VIG FOTEDAR

A THESIS
SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE
OF DOCTOR OF PHILOSOPHY
IN
MEDICAL SCIENCES

IMMUNOLOGY

EDMONTON, ALBERTA
FALL 1987

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(SIGNED) ....Rati Fotedar........

PERMANENT ADDRESS:

Apt. 1609, 11135 - 83 Avenue

Edmonton, Alberta T6G 2C6

DATED ......May 29,... 19 87

THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled *In Vitro* Mechanisms of Murine B Cell Activation submitted by Rati Fotedar in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Medical Sciences (Immunology).

....

Supervisor

T Wegmann ....

Bhagirath Singh ....

D.L.J. Tyrrell ....

Eli E. Sercarz ....

External Examiner

Date ....April 10, 1987........

Dedication

Dedicated with love and affection to my parents, Arun, and Mimi.

Abstract

In this thesis, the role of T cell- and macrophage-derived soluble factors on the activation of B cells and the relationship between early changes in ion flux and amino acid transport in cell activation is examined. A B cell activation model in which proliferation of B cells and their differentiation into antibody-forming cells (AFC) depends on a distinct set of signals, is described. B cells recognizing chicken B^2 MHC alloantigens were enriched by virtue of their property to form rosettes with chicken red blood cells (CRBC). Stimulation of these rosette-forming B cells (RFC) by a polyclonal B cell activator, lipopolysaccharide (LPS), induces them to proliferate but not to differentiate to AFC. Addition of both LPS and EL-4 supernatant (EL-4 sup) triggered proliferation and differentiation of RFC into CRBC-specific AFC. EL-4 sup alone, in the absence of LPS, was ineffective in inducing either RFC to proliferate or differentiate.

Next, the biological relevance of early changes in Na^+/K^+ ATPase and proline transport in B cells with respect to proliferation and/or differentiation, was examined. LPS activated Na^+/K^+ ATPase, enhanced the transport of proline and synthesis of RNA, protein, and DNA, without inducing differentiation into AFC. Stimulation by both LPS and EL-4 sup induced RFC to proliferate, synthesize μ mRNA, and mature into IgM-secreting cells. However, EL-4 sup, in the absence of LPS, activated Na^+/K^+ ATPase while it failed to enhance proline transport or synthesis of DNA or μ mRNA. These experiments demonstrate an increase in Na^+/K^+ ATPase activity without subsequent induction of DNA synthesis. This is in contrast to the notion that activation of the Na^+/K^+ ATPase is mandatory for cells to proceed through blast formation accompanied by DNA synthesis. Therefore, activation of Na^+/K^+ ATPase is not causally linked to B cell proliferation and/or differentiation.

Finally, the regulatory role of recombinant interleukin-2 (r IL-2) and interleukin-1 (r IL-1) in B cell activation was examined. In the presence of LPS, r IL-2 induced differentiation of RFC into AFC. This antigen-specific antibody response obtained in the presence of r IL-2 and LPS was enhanced 4 to 8 fold by the addition of r IL-1. Neither r IL-1 nor r IL-2 induced proliferation of RFC in the absence of LPS. These results demonstrate that r *IL-2 plays a direct role in differentiation of LPS-stimulated B cells, and*

r IL-1 enhances the antibody response generated by r IL-2.

Preface

Studies involving measurement of $^{86}Rb^+$ influx and amino acid transport were conducted in collaboration with Drs. G.J. Kaplan and A. Severini. Initial studies to detect μ mRNA using a μ cloned cDNA probe were done with Drs. V. Paetkau and E. Holowachuck. Some of these results have been published in the *Journal of Immunology* (136(2):562, 1986) and *Lymphokine Research* (4(4):343, 1985). Results reporting on the roles of recombinant IL-2 and IL-1 will be submitted for publication.

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I wish to thank my supervisor, Dr. Erwin Diener, for his support and enthusiasm during the course of this work. I am grateful to the members of my supervisory committee for their suggestions and criticism and to the others for providing reagents, procedures, and cheering through the rough times with good humor. C. Bleackley, A. Fu, P. Gambel, D. Gong, D. Green, I. Griffith, L. Guilbert, E. Holowachuk, J.G. Kaplan, J. Lauzon, C. Lee, P. Lomedico, P. Mannoni, S. McCarthy, S. Novak, V. Paetkau, A. Severini, C. Shiozawa, B. Singh, L. Tyrell, R. Vergidis, M. Voralia, T.G. Wegmann, H. Zheng, D. Zinyk.

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Table of Contents

Chapter Page

I. Literature Review 1

A. Introduction 1

B Lymphocyte Development 3

B Cell Heterogeneity 5

B. B Cell Activation 6

Activation of B Cells by Lipopolysaccharide 7

Activation of B Cells by Anti-Immunoglobulin Antibodies 9

One Signal Versus Two Signals 10

Other Activators of B Cells 11

C. Cellular Cooperation and Soluble Factors 12

T Cell-Mediated Triggering of B Cells 13

Antigen-Specific T Cell Helper Factors 14

Antigen-Nonspecific T Cell Helper Factors that Affect B Cell Growth and Differentiation 15

Other Factors Involved in B Cell Activation 20

Role of Antigen-Presenting Cells in B Cell Activation 23

Role of IL-1 in B Cell Activation 23

D. Molecular Mechanisms of B Cell Activation 25

Activation of Na^+/K^+ Adenosine Phosphatase (ATPase) 25

Activation of Amino Acid Transport 27

Ca^{2+} Mobilization, Phosphoinositol Turnover, and Protein Kinase C ... 27

Regulation of B Cell Response by Prostaglandins 29

Role of Cyclic Nucleotides 30

Role of G Proteins 31

II. Materials and Methods 33

III. Results 49

A. Regulation of B Cell Activation by LPS and EL-4 Sup 49

Enrichment of B Cells for Antigen-Specific Response 50

Proliferation and Differentiation of RFC Depends Upon LPS and EL-4 Sup, Respectively 50

The Antibody Response Generated by LPS and EL-4 Sup is Antigen Specific 55

Effect of Macrophage and T Cell Depletion on B Cell Activation 55

Both LPS and EL-4 Sup are Required During the Early Phase of B Cell Activation 61

IL-2- and BSF-1-Like Activity of EL-4 Sup 66

Discussion 66

B. Correlation Between Early Changes in Na^+/K^+ ATPase, Amino Acid Transport, and B Cell Activation 69

LPS and EL-4 Sup Activate $^{86}Rb^+$ Influx 69

LPS, but Not EL-4 Sup, Activates Proline Transport 78

LPS, but Not EL-4 Sup, Induces RNA, Protein, or DNA Synthesis ... 81

EL-4 Sup Induces an Increase in Cytoplasmic μmRNA, PFC, and IgM Secretion of LPS-Stimulated RFC 81

Effect of EL-4 Sup is Not Due to Contamination by PMA 88

Discussion 93

C. Role of Recombinant IL-2 and IL-1 in LPS-Dependent Activation of B Cells 100

Functional Analysis of RFC 4' 101

IL-2 Induces the PFC Response by LPS-Stimulated RFC 4' 105

IL-1 Enhances the IL-2-Induced PFC Response by LPS-Stimulated RFC 4' 105

Effect of IL-2 and IL-1 on the PFC Response by RFC 4' Depleted of T Cells and Adherent Cells 110

IL-2 and IL-1 are Both Required During the Early Phase of the B Cell Response to LPS 110

Discussion 116

IV. Summary 120

V. References 122

List of Tables

Table Page

1 Effect of EL-4 Sup on the Induction of PFC 57

2 Specificity of PFC Response with Spleen Cells 58

3 Specificity of PFC Response with T-Depleted Spleen Cells 59

4 Specificity of PFC Response with RFC 60

5 Effect of Macrophage and T Cell Depletion on Differentiation of RFC 62

6 Kinetics of Induction of Proliferation by Addition of LPS and EL-4 Sup 63

7 Effect of LPS and EL-4 Sup on the Proliferation and Differentiation of RFC 71

8 ^{86}Rb$^+$ Influx in RFC Stimulated with LPS or EL-4 Sup 74

9 ^{86}Rb$^+$ Influx and Proline Transport in Spleen Cells and Thy-1.2-Depleted Spleen Cells 75

10 Proline Transport in RFC Stimulated with LPS or EL-4 Sup 80

11 Kinetics of IgG Secretion by RFC 91

12 Spleen Cells and Thy-1.2-Depleted Spleen Cells Secrete IgM on Stimulation with LPS 92

13 Inhibition of PFC Response by PMA 94

14 ^{86}Rb$^+$ Influx in RFC 4' Stimulated with LPS or EL-4 Sup 102

15 Effect of LPS and EL-4 Sup on Proliferation and Differentiation of RFC 4' 104

16 Effect of Recombinant IL-2 on Differentiation of RFC 4' 106

17 Effect of r IL-1 on Differentiation of RFC 4' and RFC 25' by LPS and EL-4 Sup 107

18 Dose Response of r IL-1-Induced Enhancement of Differentiation 108

19 Effect of r IL-2 and r IL-1 on Differentiation of RFC 4' 109

20 Effect of LPS and EL-4 Sup on Proliferation and Differentiation of RFC 4' Depleted of Adherent and Thy-1.2-Positive Cells 111

21 Effect of r IL-2 and r IL-1 on Differentiation of RFC 4' Depleted of Adherent and Thy-1.2-Positive Cells 112

22 Dose Response Effect of r IL-2 and r IL-1 on Differentiation of LPS-Activated Cells 113

23 Effect of r IL-2 and r IL-1 on Proliferation of RFC 4' Depleted of Adherent and Thy-1.2-Positive Cells 114

24 Effect of Recombinant Mouse and Human IL-1 on Differentiation 117

List of Figures

Figure Page

1 Illustration of Rosette Preparation 51

2 Effect of LPS on Proliferation 52

3 Effect of Different Concentrations of RFC on the Activation of Proliferation Induced by LPS and/or EL-4 Sup 53

4 Effect of Different Concentrations of RFC on Generation of PFC Response with LPS and/or EL-4 Sup 54

5 Effect of EL-4 Sup on Proliferation 56

6 Effect of LPS and/or EL-4 Sup on the Viability of RFC 64

7 Kinetics of Induction of PFC by Addition of LPS and EL-4 Sup 65

8 EL-4 Sup Exhibits BSF-1-Like Activity 67

9 Activation of ^{86}Rb^{+} Influx by LPS and/or EL-4 Sup 72

10 Inhibition of ^{86}Rb^{+} Influx by Ouabain 76

11 Effect of Ouabain on LPS-Induced Proliferation 77

12 Rate of Proline Transport in RFC Stimulated with LPS and/or EL-4 Sup 79

13 Kinetics of RNA, Protein, and DNA Synthesis in RFC Activated with LPS, EL-4 Sup, or LPS and EL-4 Sup 82

14 Percentage Increase in Cell Yield of RFC Stimulated with LPS, EL-4 Sup, or LPS and EL-4 Sup 83

15 Dot-Blot Analysis of Cytoplasmic μmRNA 85

16 Kinetics of μmRNA Induction 86

17 Northern Analysis of μmRNA from RFC Activated with LPS and EL-4 Sup 87

18 Kinetics of Total and B^{2}-Specific Secretory IgM 89

19 Kinetics of B^{2}-Specific IgM-Secreting Cells 90

20 Effect of PMA on Proliferation of RFC 95

21 Effect of Different Concentrations of RFC 4' on Activation of Proliferation with LPS 103

22 Kinetics of Induction of Differentiation by Addition of LPS, r IL-2, or r IL-1 115

List of Abbreviations

A cell	accessory cell
AFC	antibody-forming cell
ASHF	antigen-specific helper factor
ATPase	adenosine phosphatase
β-ME	β-mercaptoethanol
B	B locus-chicken MHC
B cell	bone marrow-derived cell
BCDF	B cell differentiation factor
BCGF	B cell growth factor
BCL-1	B cell lymphoma-1
BMF	B cell maturation factor
BRMF	B cell replication and maturation factor
BSA	bovine serum albumin
BSF-1	B cell stimulatory factor-1
cAMP	cyclic adenosine monophosphate
cDNA	cloned deoxyribonucleic acid
cGMP	cyclic guanosine monophosphate
Con A	Concanavalin A
CRBC	chicken red blood cells
CSF	colony-stimulating factor
DAG	diacylglycerol
dCTP	deoxycytosine triphosphate
DDW	double-distilled water
DNA	deoxyribonucleic acid
DPC	diethyl pyrocarbonate
$DxSO_4$	dextran sulphate
EDTA	ethylenediaminetetraacetic acid
EGTA	ethyleneglycol-bis-(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid

EL-4 sup	EL-4 supernatant
FCS	fetal calf serum
γ	IgG
H chain	heavy chain
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
Ia	I region-encoded proteins
Ig	immunoglobulin
IFN	interferon
IL-1	interleukin 1
IL-2	interleukin 2
IP_3	inositol triphosphate
J chain	joining chain
L chain	light chain
LPS	lipopolysaccharide
μ_m	membrane IgM
μ_s	secreted IgM
MHC	major histocompatibility complex
mRNA	messenger ribonucleic acid
NP-40	Nonidet P-40
PBS	phosphate-buffered solution
PE	phorbol esters
PFC	plaque-forming cells
PG	prostaglandin
PGE_2	prostaglandin E_2
PHA	phytohaemmaglutinin
pI	isoelectric point
PI	phosphoinositol
PKC	protein kinase C
PMA	phorbol 12-myristate 13-acetate

PWM	pokeweed mitogen
r IFN	recombinant interferon
r IL-1	recombinant interleukin-1
r IL-2	recombinant interleukin-2
RFC	rosette-forming cell
RFC 4'	rosette-forming cells obtained by 4 min final centrifugation
RIA	radioimmunoassay
RNA	ribonucleic acid
RPMI	Roswell Park Memorial Institute
SAC	*Staphylococcus aureus* Cowan I
SDS	sodium dodecyl sulphate
SRBC	sheep red blood cells
SSC	saline sodium citrate
T cell	thymus-derived cell
T_h cell	T helper cell
TCA	trichloroacetic acid
TD	thymus-dependent
TE	Tris-EDTA
TI	thymus-independent
TRF	T cell-replacing factor
V region	variable region
V_H	variable region of immunoglobulin heavy chain

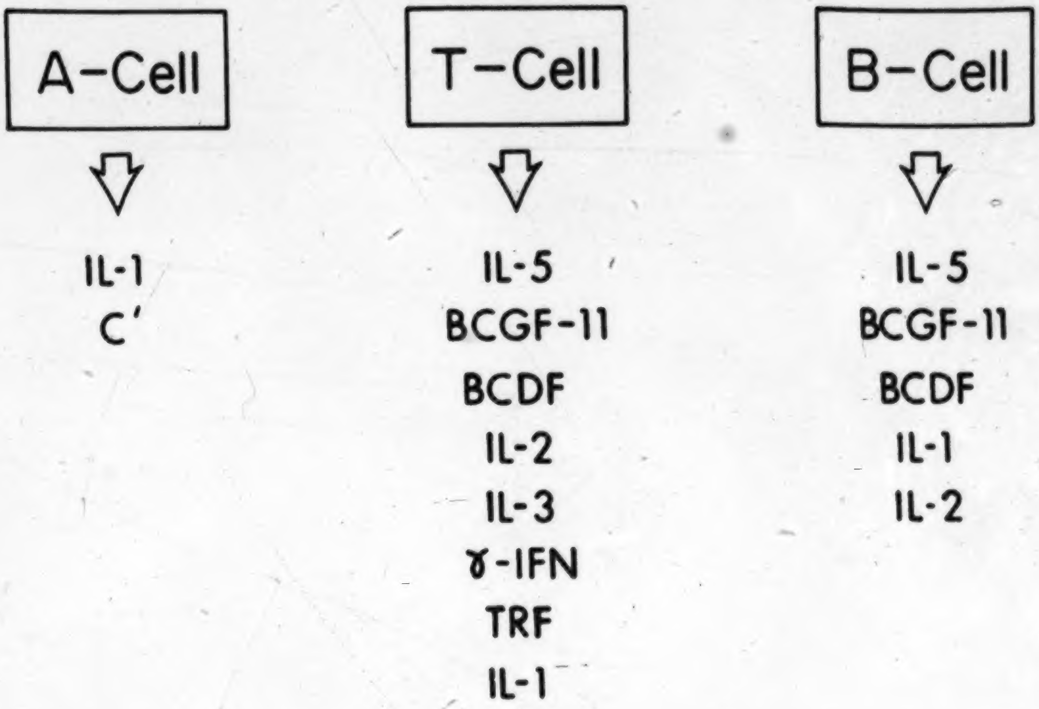
I. Literature Review

A. Introduction

The proliferation of antigen-specific bone marrow-derived lymphocytes (B cells) and their maturation into antibody-secreting cells constitutes the humoral response of the immune system to antigen (Burnet, 1957). The antigen selects those resting B cells that express one of the diverse sets of variable regions (V regions) of the heavy chains (H chains) and light chains (L chains) on their surface. Stimulation of B cells leads to several rounds of cell division (Andersson *et al*, 1977a). Maturation of B cells progresses with an increase in rate of immunoglobulin (Ig) synthesis, secretion of Ig, and, subsequently, progresses to class switching the H chain (Andersson *et al*, 1978b). After antigen stimulation, clonal expansion of B cells is accompanied by a high rate of somatic mutation in Ig genes (Tonegawa, 1983). Within an expanding clone of B cells, some cells may return to rest and remain in the immune system as long-term memory cells (Sprent, 1977).

B cell activation to most antigens is under multiple control and requires cooperation among helper T lymphocytes (T_h cells), accessory cells (A cells), and B cells (Claman *et al*, 1966; Davies *et al*, 1967; Mosier, 1967; Mitchell and Miller, 1968). B cell subpopulations differ in their genetic requirement for activation by T_h cells. Some B cells require major histocompatability complex (MHC)-restricted interaction with T_h cells while others can be activated by T_h cells in an MHC-unrestricted manner (Singer *et al*, 1979,1982). Some of the lymphokines and monokines secreted as a result of interaction between A cells, B cells, and thymus-derived lymphocytes (T cells), stimulate B cell proliferation and maturation. There is considerable confusion regarding which of these cytokines are active on B cells, their function, and the order in which they are required to stimulate B cells. A list of lymphokines/monokines is given on p. 2.

Early changes in biochemical parameters of B cells have been observed after B cell activation. These changes include an increase in Ca^{2+} (Monroe and Cambier, 1983), an increase in K^+ (Quastel and Kaplan, 1970), depolarization of surface membranes (Monroe and Cambier, 1983), changes in phosphoinositol (PI) metabolism (Coggeshal and Cambier, 1984),


A-Cell
IL-1
C'
T-Cell
IL-5
BCGF-11
BCDF
IL-2
IL-3
γ-IFN
TRF
IL-1
B-Cell
IL-5
BCGF-11
BCDF
IL-1
IL-2

Lymphokines produced by different cells. Most of this information is derived from established or transformed cell lines, hybridomas, or tumors, and very little is known for normal resting or activated cells.

IL: Interleukin
BSF-1: B cell stimulatory factor-I
BCGF-II: B cell growth factor-II
BCDF: B cell differentiation factor
γIFN: Gamma interferon
TRF: T cell-replacing factor
C': Complement components

increased I region-encoded protein (Ia) expression (Cambier and Monroe, 1985), and other surface marker expression (Freedman *et al*, 1984; Kehrl *et al*, 1984; Teddler *et al*, 1985). At the present time, we are unable to causally link a given early change in B cells to proliferation and/or maturation.

A review on B cell stimulation as it relates to the overall objectives of my thesis is given below.

B Lymphocyte Development

The earliest stage of B cell development involves commitment of a pluripotent hematopoietic stem cell to the B cell pathway and subsequent rearrangement of Ig genes and expression of cell surface Ig to serve as antigen-specific receptors. Stem cell commitment to the B cell lineage occurs in the bone marrow of adult animals and in the liver and spleen of fetuses. The very early stages of this process are poorly understood, primarily due to the heterogeneity of the precursor cell populations and the lack of suitable markers by which to distinguish and isolate these cells. As a committed cell differentiates into a pre-B cell, there is μ H chain gene rearrangement, transcription, translation, and expression of cytoplasmic μ chains, but no expression of L chain (Burrows *et al*, 1979; Siden *et al*, 1979; Levitt and Cooper, 1980). A possible late pre-B cell developmental stage in pre-B cell lines has been identified as having functionally rearranged L chain genes but no L chain messenger ribonucleic acid (mRNA) (McKenzie and Potter, 1979; Perry and Kelley, 1979). Ontogenic studies suggest that the transit time through the pre-B cell compartment is approximately 5 days in mouse fetal liver and 4 days in bone marrow. As a pre-B cell develops into an immature B cell, L chains are expressed and IgM monomers (H_2L_2) are displayed on the cell surface as antigen receptors (Vitetta and Uhr, 1975). The immature B cell matures into a B cell that co-expresses IgM and IgD on its surface (Goding, 1978). Co-expressed μ and δ chains in IgM and IgD have the same V region, and their expression exhibits allelic exclusion (Coffman and Cohn, 1977; Raschke, 1978; Herzenberg *et al*, 1982). Up to this stage, B lymphocyte development is antigen-independent, although immature B cells that express only surface IgM are capable of being affected, but not fully triggered, by contact with antigen or anti-idiotypic antibody. Further maturation of B cells requires activation by mitogen or an appropriate combination of antigen, T cells or T cell-factors, and macrophages (McKenzie and Potter, 1979; Melchers *et al*, 1980b). Once activated, B cells either mature into memory B cells, terminally differentiate into IgM-secreting plasma cells, or undergo class switching. As a result of H chain "class switching" (Davis *et al*, 1980; Yamawaki-Kataoka *et al*, 1980; Honjo *et al*, 1981; Yagi and Koshland, 1981), the same H chain variable region (V_H region) expressed on the surface of immature B cells (with constant region $C\mu$) is later expressed with other C_H regions ($C\gamma$, $C\alpha$, $C\delta$).

All classes of membrane-bound Ig molecules, including IgM and IgA, are present as monomeric structures (Melchers *et al*, 1975b). Like other integral membrane proteins, membrane μ (μ_m) chains possess hydrophobic carboxy terminal sequences (Vassalli *et al*, 1979; Parkhouse *et al*, 1980). The secreted μ (μ_s) chains, on the other hand, have hydrophilic carboxy terminal sequences. Similar studies have shown different membrane and secreted species for murine γ_3, γ_1, γ_{2a}, γ_{2b}, δ and α H chains (Wall and Kuehl, 1983).

Membrane and secreted Ig H chains are encoded by H chain mRNA species with different 3' ends generated by ribonucleic acid (RNA) processing (in Wall and Kuehl, 1983). Analysis of μ mRNA revealed that two prominent μ mRNA's, one 2.7 kb long, encoding for μ_m chains, and another at 2.4 kb, encoding for μ_s chains. Both μ mRNA species are identical through the end of the $C\mu$ constant region encoding sequence but contain different carboxy terminal and 3'-untranslated regions. The membrane and secreted Ig H chains are generated by differential RNA splicing (Early *et al*, 1980; Kehry *et al*, 1980; Rogers *et al*, 1980; Singer *et al*, 1980).

Most Ig, except IgM and IgA, is secreted as a four-chain disulphide-linked monomer (H_2L_2). IgM and IgA carry a short carboxy-terminal sequence on H chains, which permits polymerization of the monomers. These polymeric Ig molecules are covalently linked by disulfide bonds from their H chain carboxy-terminal segments to a single joining chain (J chain) per IgM or IgA polymer (Koshland, 1975; McHugh *et al*, 1981). There is also a differential expression of J chain during B cell development. J chain expression is prominent in all plasma cells and in all B cells that secrete Ig, but is absent in pre-B cells, immature B cells or pre-B cells (Raschke *et al*, 1979; Mather *et al*, 1981; McHugh *et al*, 1981).

B Cell Heterogeneity

During the normal development of the fetus, functional B cells capable of responding to particular antigens and mitogens appear in a predictable order (Gronowicz *et al*, 1974). There is increasing support accumulating in favor of the proposed existence of functionally distinct B cell subsets. In general, these subsets have been considered representative of different developmental stages of the B cell lineages; however, arguments have been advanced for distinct B cell lineages (Tittle and Rittenberg, 1980; Wortis *et al*, 1982; Hardy *et al*, 1984).

Murine B cells have been classified into subsets that differ with respect to surface marker phenotype, tissue distribution, and functional responsiveness to a variety of thymus-dependent (TD) and thymus-independent (TI) antigens (Mond *et al*, 1978; Tittle and Rittenberg, 1980; Singer *et al*, 1981; Huber, 1982; Wortis *et al*, 1982; Hayakawa *et al*, 1983; Hardy *et al*, 1984). The CBA/N mouse, like the newborn normal mouse, lacks a functional subset of mature B cells. Five differentiation markers, Lyb 3, Lyb 5, Lyb 7, M locus, and Ia.W39, all of which are absent from B cells in newborn normal and adult mice carrying the CBA/N mutation, have been identified. The mutation impairs B lymphocyte's ability to produce antibody in response to TI type-2 antigen (Scher *et al*, 1975; Mond *et al*, 1978; Huber, 1982) and results in a failure to produce at least some sets of high-affinity antibodies (Huber, 1982). CBA/N mice also have reduced responsiveness to lipopolysaccharide (LPS) (Huber and Melchers, 1979). Subnormal levels of IgG_3 antibody-producing cells were found in responses to polyclonal B cell activators such as LPS, pokeweed mitogen, and *C. parvum*, as well as to different types of antigens (McKearn *et al*, 1982). IgG_1, IgG_{2a}, IgG_{2b}, and IgA AFC responses were normal in these mice. These results suggest the existence of at least two major B cell subsets, each displaying preferences in response to polyclonal B cell activators or different types of antigen. Responses within these subpopulations generate different Ig subclasses.

Singer *et al* (1981) demonstrated that at least two distinct subpopulations of B cells exist that are distinguished by the effects of the CBA/N mutation, by their expression of

Lyb 5 determinants, and because they fundamentally differ in their genetic requirements for activation by T_h cells. The Lyb 5 determinant itself is not important in these responses. Lyb 5^+ B cells are activated by antigen-nonspecific and MHC-unrestricted factors secreted by antigen-specific and MHC-restricted T_h cells. Consequently, Lyb 5^+ B cells do not require direct physical interaction with T_h cells, whereas Lyb 5^- B cells do (Singer *et al*, 1982). Soluble anti-Ig induces an increase in size and expression of surface Ia without proliferation (Mond *et al*, 1983) in Lyb 5^+ B cells. Although anti-Ig also increases the expression of surface Ia on B lymphocytes from CBA/N mice, it is not as marked as that seen on normal cells.

Another B cell subset, Ly-1 B cells, express Ly-1 surface antigens in conjunction with IgM, IgD, Ia, and other typical B cell surface markers (Hayakawa *et al*, 1984). Ly-1 B cells possess properties of a distinct lineage that fails to develop from bone marrow-based progenitors to other B cells (Hayakawa *et al*, 1985). In addition, Ly-1 B cells are distinct from other cells in that they rarely participate in IgM antibody responses to exogenous antigens and frequently contribute to IgM autoantibody production (Hayakawa *et al*, 1983,1985).

B. B Cell Activation

The frequency of B cells capable of responding to any antigen is of the order of 10^{-4} or 10^{-5}. Thus, polyclonal activators have been of great value in studying B cell activation (McConnell, 1975). Polyclonal activators stimulate a high proportion (up to 70 or 80%) of all B cells. The gross morphological changes and biochemical events occurring in polyclonally stimulated lymphocytes resemble many of the antigen-induced immune reactions. Furthermore, B cells stimulated by suitable polyclonal activators are capable of synthesizing Ig in a fashion similar to cells stimulated by antigens. LPS and anti-Ig function by cross-linking membrane receptors on B cells. While LPS triggers B cells by circumventing Ig receptors on the cell (Coutinho and Möller, 1974), anti-Ig cross-links Ig receptors and induces only polyclonal proliferation. These two activities will be discussed in more detail.

Activation of B Cells by Lipopolysaccharide

LPS extracted from gram-negative bacteria has been demonstrated to be a potent mitogen for murine B lymphocytes (Andersson *et al*, 1972). The discovery that LPS could activate a substantial proportion of B lymphocytes in culture to proliferate and mature with concomitant production of Ig, represents an important contribution to the study of lymphocyte biochemistry (Greaves and Jannossy, 1972; Andersson *et al*, 1977a). Later experimental evidence has shown that mitogenic activity of LPS is dependent on the presence of the lipid region (lipid A) of the molecule (Andersson *et al*, 1973). However, the precise nature of the interaction between LPS and the cells it stimulates remains elusive. Mutant C3H/HeJ (Sultzer, 1976) and C57BL/10ScN mice (Coutinho and Mao, 1978) possess cells that are unresponsive to stimulatory effects of the lipid A component of LPS but respond normally to lipoprotein (Melchers *et al*, 1975c). The mutation in these strains has been associated to a single gene on chromosome 4 (Coutinho and Mao, 1978; Watson *et al*, 1978). It has been speculated that the gene product of this locus may be the cell surface receptor for LPS. Jakobovits *et al* (1982) used Sendai virus envelopes as vehicles to insert plasma membrane components of B cells from the LPS responder strain into murine T cells, or into B cells derived from C3H/HeJ mice. They observed that, through this procedure, cells aquired LPS responsiveness, thus indicating that the aberrant behavior of C3H/HeJ B cells to LPS was due to lack of a suitable membrane constituent.

Using antiserum raised against the LPS receptor, Forni and Coutinho (1978) demonstrated the presence of LPS "receptors" on the membrane of a proportion of B cells from responder mice. Watson *et al* (1980), however, failed to reproduce these results. In spite of this provocative evidence in support of a receptor-mediated interaction of LPS with lymphocytes, the existence of such a structure remains controversial. LPS was found to bind to B lymphocytes from LPS responders more significantly than to LPS nonresponders (Chaby *et al*, 1984).

Approximately one in three splenic B cells from a variety of mouse strains are stimulated by LPS to clonal replication and IgM secretion (Andersson *et al*, 1977b). The mitogen has been shown to be required in G_1 phase to initiate the cell cycle (Melchers *et al*, 1980b). Upon withdrawal of LPS, immunoblasts once generated continued to secrete Ig for several days (Andersson *et al*, 1977b). Mitogen-stimulated B cells can be propagated *in vitro* as continuous lines for up to 2-3 weeks (Melchers *et al*, 1975a).

LPS has been implicated to control Ig expression at three distinct levels. First, LPS has been suggested to increase Ig gene transcription. This was shown by its effect on normal splenic B cells (Yuan and Tucker, 1984a,b; Lamson and Koshland, 1984). LPS induced a large increase in the steady-state level of μ mRNA. Pre-B cell line 70Z/3 contains a functionally rearranged K chain gene that is transcribed only on stimulation with LPS. This suggests a direct effect of LPS on Ig gene transcription (Maki *et al*, 1980). Secondly, LPS has been suggested to control the post-transcriptional stage of Ig expression. LPS treatment of the B cell lymphoma-1 (BCL_1) line does not change the level of μ transcription but increases μ_s mRNA several fold over μ_m mRNA (Yuan and Tucker, 1982). Similarly, stimulation of normal B cells by LPS does not decrease δ_m mRNA transcription levels below that of normal B cells but δ polypeptide synthesis is completely shut down. This suggests that post-transcriptional events further regulate the formation of δ_m mRNA (Yuan and Tucker, 1984b). Sitia *et al* (1985) have indicated that in murine B lymphoma, LPS induces an increase in secreted α-chain.

Finally, LPS treatment can modulate isotype switching, although the molecular events involved are poorly understood. Secretion of IgG can be induced by LPS in murine B cells *in vitro* (Kearney and Lawton, 1975; Andersson *et al*, 1978a; Gronowicz *et al*, 1979a), and requires several rounds of cell division (Zauderer and Askonas, 1976; Gronowicz *et al*, 1979b). Radbruch and Sablitzky (1983) treated normal splenic B cells with LPS and observed a switch from IgM to IgG_3. When IgG_3-secreting cells were purified, these authors found that deoxyribonucleic acid (DNA) rearrangements and deletion of $C\mu$ segments had occurred. Yuan and Vitetta (1983) noted that B cells at low density in the presence of LPS predominantly express and secrete IgG_3 with little or no expression of $mIgG_{2b}$ or $mIgG_1$. However, addition of T cell-derived B cell differentiation factor (BCDFγ)-containing PK 7.1 supernatant was necessary to induce a switch from IgG_3 to IgG_1 secretion accompanied by an increase in mRNA for γ_1 (Isakson *et al*, 1982). Forni and Coutinho (1982) argue that although IgG_1 is expressed on LPS-stimulated B cells, IgG_3 is the predominant form of secretory IgG.

Activation of B Cells by Anti-Immunoglobulin Antibodies

Anti-Ig antibodies have been used to activate B lymphocytes since the initial observation that anti-allotype antibody induces the proliferation of rabbit peripheral blood lymphocytes *in vitro* (Sell and Gel, 1965). Although some antisera activate B cells, others inhibit B cell activation by antigen or mitogen *in vitro* (Cooper, 1980). Activation of murine B cells was efficiently induced both by intact anti-Ig antibodies and $F(ab')_2$. However, intact anti-Ig antibodies sometimes failed to activate B cells, apparently because Fc binding leads to a negative B cell signal (reviewed by Braun and Unanue, 1980). Removal of the Fc portion of the anti-Ig molecules or inhibition of their binding to Fc receptors relieves the block and permits B cell activation. Antibodies which did not activate B cells in soluble form could do so if coupled to an insoluble matrix (Parker, 1975; Parker *et al*, 1979). B cells cultured with relatively low concentrations of anti-IgM, enter G_1 phase of the cell cycle (Defranco *et al*, 1982a) accompanied by a rapid increase in volume (Defranco *et al*, 1982b), RNA content (Defranco *et al*, 1982a; Monroe and Cambier, 1983), and surface Ia, without entering into the S phase (Mond *et al*, 1981). To enter S phase, the B cells require either a high concentration of anti-IgM or a low concentration of anti-IgM together with T cell-derived factors (Howard *et al*, 1982). B cells stimulated by whole anti-Ig or $F(ab')_2$ acquire the ability to proliferate in response to T cell-derived factors (Proust *et al*, 1985).

Monoclonal anti-μ antibodies theoretically offer distinct advantages over heterologous anti-μ antibodies prepared for the study of B cell triggering. Activation of purified small B cells by monoclonal anti-Ig provides support for the idea that B cell activation and proliferation are the direct consequence of cross-linkage of the membrane-bound Ig molecules. Initially, the mitogenic activity of monoclonal anti-μ antibody was reported to be very feeble (Godal *et al*, 1983; Kuritani and Cooper, 1983; Julius *et al*, 1984; Leptin *et al*, 1984). Later, the *in vitro* triggering of human B cell proliferation by monoclonal anti-μ antibodies was shown to require a crucial threshold density of small B cells (Maruyama *et al*, 1985). In contrast, large B cells were unresponsive to the monoclonal anti-μ antibodies and these antibodies were capable of inhibiting T cell factor-induced differentiation into IgM plasma cells (negative signals). Other monoclonal antibodies specific for murine IgM which did not activate B cells in soluble form, did so when coupled to an insoluble matrix (Leptin, 1985). Murine B cell proliferation can be achieved by a high concentration of soluble monoclonal antibody (Julius *et al*, 1984) in the presence or absence of T cell-derived factor (BSF-1) (Nakanishi *et al*, 1984).

One Signal Versus Two Signals

Several hypotheses of the B cell-triggering mechanism emphasize the role played by the interaction of antigenic determinants with specific Ig receptors on the B cell surface (reviewed by Barton and Diener, 1975). Two hypotheses that have been much discussed and debated are the two-signal hypothesis of Bretscher and Cohn (1970) and the one-signal hypothesis of Coutinho and Möller (1974). Bretscher and Cohn (1970) suggest that two signals are required for a positive B cell response. Signal 1 is delivered by the contact of haptenic determinants with B cell receptor (Ig) sites and signal 2 is provided by interaction of a carrier determinant with a specific T cell-derived helper factor. Signal 1 alone induces a tolerogenic state unless counteracted by signal 2. While the two-signal hypothesis explains the capacity of nonimmunogenic carrier molecules to induce hapten-specific B cell tolerance (Katz *et al*, 1975), it does not account for the action of TI antigens.

The one-signal hypothesis of Coutinho and Möller (1974) is derived from the study of polyclonal activation by mitogens such as LPS. It suggests that Ig receptors serve only to focus antigen onto a specific B cell, whereas the actual triggering of B cells in the case of TI antigens, is accomplished by interaction of the cell with the nonspecific mitogenic determinant in the antigen molecule. The response to TD antigens is mediated by nonspecific mitogenic factors produced by T cells, which are focussed onto relevant B cells as a result of antigen bridging between B and T cells. This hypothesis explains the mitogenic effect of LPS and accounts for the existence of nonspecific stimulatory T cell factors, although it does not account for the action of the antigen-specific T cell factor (see Taussig, 1980).

While the above hypotheses are successful in rationalizing certain aspects of B cell behavior, it is difficult to understand the involvement of MHC-encoded gene products in T-B cell collaboration (Katz *et al*, 1973), the effect of distinct B cell subpopulations (Singer *et al*, 1981), and the role of antigen-specific (Taussig, 1980) T helper factor in terms of the above models.

Other Activators of B Cells

Most mitogen-activated B cell blasts are reactive to more than one mitogen, suggesting that multiple-mitogen receptors may exist on single B cells (Andersson *et al*, 1977b). The purified protein derivative of tuberculin stimulates resting B cells to mature into Ig-secreting cells in the absence of replication (Sultzer, 1976). Dextran sulphate ($DXSO_4$) is a polyclonal B cell activator that is particularily active on immature B cells (Gronowicz *et al*, 1974). Recent studies show that $DXSO_4$ induces 5-40% of normal splenic B cells to enter G_1 phase during 48 hr of culture without significant cell division, a situation similar to that discussed for anti-Ig stimulation. There is a synergy between LPS and $DXSO_4$ such that virtually all B cells can be induced to form clones in the presence of both mitogens (Wetzel and Kettman, 1981). Concanavalin A (Con A), a plant lectin derived from *Canavalia ensiformis*, induces DNA synthesis in mouse T, but not B, lymphocytes, although it binds equally well to both cell types (Stobo *et al*, 1972). Con A can directly induce B cell proliferation when presented in an insolubilized form, for example, when bound to sepharose beads (Greaves and Janossy, 1972; Andersson *et al*, 1972). Recent reports indicate that Con A, at concentrations which are optimal for the T cell response, induces purified murine B cells to depolarize, enlarge, and display increased levels of Ia antigen, although it does not induce B cells to divide (Hawrylowicz and Klaus, 1984).

There have been reports that *Corynebacterium parvum*, a potent stimulator of the reticuloendothelial system, is an effective B cell activator (Howard *et al*, 1973; McKearn *et al*, 1982). Much less is known about mitogenic properties of PWM (Waxdal and Basham, 1974; McKearn *et al*, 1982). PWM stimulates both T and B lymphocytes to mitosis. B cells can be induced to secrete antibody in the presence of T cells or macrophages (Parkhouse *et al*, 1972; Piquet and Vassalli, 1973; Quintas and Lefkovitz, 1974).

Experiments comparing the ability of various B cell activators (LPS, *C. parvum*, and PWM) to stimulate B cells to produce antibodies, were described by McKearn *et al* (1982). LPS was shown to induce IgG_{2b}- and IgG_3-dominant plaque-forming cell (PFC) responses. PWM induced significant increases in the levels of all four IgG subclasses, especially IgG_1 and IgG_3. Responses to *C. parvum* were similar to those elicited by PWM. Of the four subclasses of IgG expressed in mouse, IgG_3 was the only subclass shared in responses to all three polyclonal B cell activators.

Phorbol esters (PE) are low-molecular-weight lipophilic substances that have tumor-promoting activity. These compounds produce a variety of effects on normal cellular function, most likely mediated by their ability to activate protein kinase C (PKC) (Castanaga *et al*, 1982). While the mitogenic activity of PE on T lymphocytes is well documented, less is known about their effect on B cells. Phorbol 12-myristate 13-acetate (PMA) has been shown to be mitogenic for human lymphocytes (Roth *et al*, 1982) but surprisingly, it was not mitogenic for rodent lymphocytes (Abb *et al*, 1979). PMA induces membrane depolarization, an increase in Ia expression, and the transition of cells from G_0 to G_1 phase similar to that known to occur during mitogen, anti-Ig, and antigen activation of murine B cells (high doses of PMA, i.e., 10 to 100 ng/ml, are required for equivalent depolarization to levels observed by anti-Ig treatment). Additional signals provided by growth factors may be required for thymidine incorporation (Monroe *et al*, 1984). Addition of PMA to B cells inhibits LPS-induced IgM secretion but not proliferation. The concentration of PMA necessary to induce this effect is very low (10 pM) (Isakson and Simpson, 1984). From a technical standpoint, caution should be exercised in interpreting results of experiments in which PMA is utilized to generate lymphokine-containing supernatant, such as EL-4 supernatant (EL-4 sup).

C. Cellular Cooperation and Soluble Factors

The B cell response normally follows an encounter with antigen and, in most cases, antigen alone is insufficient to trigger the response. B lymphocyte subpopulations differ in their requirements for stimulation by TD and both types 1 and 2 TI antigens (Lewis and Goodman, 1977; Mosier *et al*, 1977; Mond *et al*, 1978; Tittle and Rittenberg, 1980). This distinction between TD and TI antigens was made on the basis of differential requirement for T cells to generate an antibody response. However, it has become clear that responses to TI antigens can, nevertheless, be influenced by T lymphocytes as well as T lymphocyte-derived factors (discussed in *Chapter I*; Mond *et al*, 1980; Yaffe *et al*, 1981). Another cell which plays an important role in relation to B lymphocyte activation is the adherent cell. This has been observed in polyclonal, mitogenic responses (Chused *et al*, 1976; Nordin, 1978; Hoffmann *et al*, 1979; Boswell *et al*, 1980; Hoffmann, 1980; Martinez-Alonso *et al*, 1980; Corbel and Melchers, 1983; see Corbel and Melchers, 1984). The following section includes a discussion on the soluble factors produced by T lymphocytes and macrophages and their influence on growth and maturation of B cells.

T Cell-Mediated Triggering of B Cells

The first insight of the mechanism by which T_h cells and B cells interact was provided by Rajewsky *et al* (1969), Raff (1970), and Mitchison (1971). While analyzing the anti-hapten antibody response stimulated by carrier hapten conjugates, these investigators observed that optimal secondary anti-hapten antibody responses required that T cells be previously primed to carrier, and the B cells to hapten. Effective T_h-B cell collaboration involved physical joining of a T_h cell with B cell *via* an antigen bridge.

In addition to the carrier specificity, T_h cells recognize determinants encoded by class II MHC genes. The interaction between T_h cells and antigen-presenting cells is MHC-restricted (Erb and Feldmann, 1975; Katz and Benacerraf, 1975). However, the cellular interaction between T_h cells and B cells may be either MHC-restricted (Katz *et al*, 1973; Sprent, 1976; see Singer and Hodes, 1983) or MHC-unrestricted (Singer *et al*, 1979,1982).

Cognate interaction refers to the recognition of both antigen and class II MHC products on the B cell surface by T_h cells as a requirement for activation. As mentioned above, while some B cells participate in cognate cellular interaction with

T_h cells, others can be stimulated by several MHC-unrestricted nonspecific lymphokines. The next section describes antigen-specific and antigen-nonspecific T cell lymphokines.

Antigen-Specific T Cell Helper Factors

The role of antigen-specific T cell-replacing factors (TRF) in immune regulation has been demonstrated (reviewed by Taussig, 1980; Feldmann and Kontiainen, 1981). The functional and genetic properties of these molecules have been extensively studied by establishing a number of T cell hybridomas producing these T cell factors (Mozes and Haimovich, 1979; Eshhar *et al*, 1980; Apte *et al*, 1981; Lonai *et al*, 1981; DeKruyff *et al*, 1983; Lifshitz *et al*, 1983; Miyatani *et al*, 1983; Shiozawa *et al*, 1984). Nevertheless, it has been difficult to generate antigen-specific helper factor (ASHF)-producing T cell hybrids. Most such hybridomas produce relatively small amounts of helper factor, and biochemical characterizations of these helper factors have been slow. One such factor was isolated by affinity chromatography of supernatants from an *in vitro* antigen-activated helper T line (Lifshitz *et al*, 1983). This factor exhibited antigen-specific helper activity similar to intact T_h cells. In another approach, the isolation of ASHF has been facilitated by affinity chromatography using anti-ASHF monoclonal antibodies (Hiramtsu *et al*, 1982). Using these monoclonal antibodies, KLH-specific T helper factor was isolated from lysates of biosynthetically-labelled T cell hybridoma FL10 (Miyatani *et al*, 1983). Besides exhibiting exquisite specificities for the antigen with which they were generated, ASHF also possess determinants controlled by Ig V_H region and H-2 (for review see Tada and Okumura, 1979). It is suggested that specific T cell factors may be secreted receptors of T cells (Cone *et al*, 1983; DeKruyff *et al*, 1983). However, whereas the function of most T cells is H-2-restricted, many antigen-specific T cell factors are not H-2-restricted. An antigen-specific, T cell-derived helper factor recognizing alloantigens on chicken red blood cells (CRBC) was partially-purified from antigen-primed T_h cells. This factor induces antigen-dependent B cell proliferation and differentiation in the presence of non-antigen-specific lymphokines (Shiozawa *et al*, 1984) and was MHC-restricted. Such restriction was shown to involve B cells and not macrophages (Shiozawa *et al*, 1980). Lonai *et al* (1981) described an H-2-restricted, chicken gamma globulin-specific helper factor from cloned hybridoma cells that possessed determinants shared with the Ig V_H region. This factor provided "helper" activity for B cells that shared H-2K and I-A antigens with the hybridoma cells.

Thus, antigen-specific T cell factors appear to interact with B cells in an MHC-restricted manner, analogous to that demonstrated for T_h-B cell interaction. The behavior of antigen-specific T_h cell factors for activating distinct B cell subpopulations such as those represented by Lyb 5^- and Lyb 5^+ cells, remains to be examined. The "physiological" role of such factors in the co-operation of T_h-B cells remains a reasonable assumption.

Antigen-Nonspecific T Cell Helper Factors that Affect B Cell Growth and Differentiation

The possibility that the helper functions of T cells may be mediated by soluble products released from T cells was originally proposed by Dutton and his colleagues (1971,1975). This was confirmed by Schimpl and Wecker (1972), who showed that the active soluble factor(s) in culture supernatants of mitogen- or antigen-stimulated T cells induced antibody secretion by acting on B cells already proliferating in response to antigen. These studies led to the speculation that B cells could be activated into antibody-secreting cells by binding of a given antigen to Ig receptors and T cell-derived antigen-nonspecific helper factor(s) (Parker *et al*, 1979; Isakson *et al*, 1981; reviewed in Kishimoto, 1985). Polyclonal stimulation of B cells by anti-Ig antibodies, unlike LPS, failed to induce the differentiation of B cells to Ig synthesis and secretion (Parker *et al*, 1979). The failure to induce Ig secretion was overcome by addition of a cell-free supernatant from Con A-stimulated spleen cells possibly containing TRF.

T cell factor-dependent B cell activation can be divided into two stages: a growth factor-dependent proliferation and a differentiation factor-dependent maturation into Ig-secreting cells. T cell factors involved in proliferation and differentiation of activated B cells are described below.

Murine B cell stimulatory factor-1 (BSF-1) was originally identified as a T cell-derived product which synergizes with affinity-purified anti-IgM antibodies (co-stimulation) to induce polyclonal proliferation of B lymphocytes (Howard *et al*, 1982). The same factor also supports the proliferation of LPS-activated B cell blasts (Leanderson *et al*, 1982). This factor was present in the culture supernatants of PMA-stimulated EL-4 thymoma (Howard *et al*, 1982), in supernatants of a variety of T cell hybridomas (Leanderson *et al*, 1982; Lernhardt *et al*, 1982), alloreactive T cell clones (Puré *et al*, 1983) and is produced by long-term lines of normal mouse T cells in response to antigenic stimulation (Howard and Paul, 1983a). A human counterpart of this factor induces DNA synthesis in normal or leukemic B cells stimulated with anti-Ig antibodies, anti-idiotypic antibodies, or the polyclonal activator *Staphylococcus aureus* Cowan I (SAC) (Maizel *et al*, 1982; Muraguchi *et al*, 1982; Yoshizaki *et al*, 1982). Murine and human BSF-1 are glycoproteins (Farrar *et al*, 1983) with similar molecular weights (18-20 kD) as determined by gel filtration (Howard *et al*, 1982; Yoshizaki *et al*, 1982; Butler *et al*, 1983). BSF-1 is readily separable from many other lymphokines, for example, interleukin-2 (IL-2) and colony-stimulating factor (CSF) (Mizel, 1982; Farrar *et al*, 1983), B cell growth factor (BCGF)-II and B151-TRF (Howard *et al.*, 1984), by varying the concentration of ethanediol required for elution from a phenyl sepharose column.

Direct binding of BSF-1 to the surface of B cells is indicated by absorption studies showing depletion of activity in supernatants incubated with LPS-activated murine B cells (Leanderson *et al*, 1982) or with anti-Ig or anti-idiotype-activated human B cells (Yoshizaki *et al*, 1982). While BSF-1 itself does not lead to Ig synthesis (Howard *et al*, 1982), it is required for anti-Ig-induced antibody-forming cell (AFC) responses (Nakanishi *et al* 1983) and in antibody responses to certain antigens. Kinetic analysis has shown that BSF-1 is required very early (G_1 phase) in the B cell response induced by anti-IgM antibodies (Howard and Paul, 1983a). B cell populations cultured for 24 hr with BSF-1 in the absence of anti-IgM, display substantial increases in cell volume (Thompson *et al*, 1985) and in expression of class II MHC molecules (Roehm *et al*, 1984; Noelle *et al*, 1984) and genes (Noelle *et al*, 1986). A variety of other lymphokines, including IL-2, interleukin-1 (IL-1), interferon (IFN)-γ, BCDFμ, and BCGF-II, failed to induce an increase in expression of Ia on resting B cells (Oliver *et al*, 1986). Furthermore, Rabin *et al* (1985) showed that 24 hr preculture of B lymphocytes with BSF-1 speeds the entry into S phase of B cells subsequently cultured with anti-IgM and BSF-1. B cell blasts and large B cells do not enter S phase in response to BSF-1. These results strongly suggest that BSF-1 acts upon resting B cells and, thus, it should be regarded as a B cell activation factor and not a BCGF.

Ohara and Paul (1985) purified BSF-1 to homogeneity using monoclonal antibodies to BSF-1. This protein exhibited co-stimulator activity with anti-Ig and BCDFγ activity. It also induces class II MHC molecules on B cells (Oliver *et al*, 1985) enhances the IgE response of LPS-activated B cells (Coffman *et al*, 1986), and induces the proliferation of the IL-2-dependent T_h cell line, HT2 (Fernandez-Botran *et al*, 1986). These results suggest that the same or very closely related lymphokine(s), mediate multiple activities.

Recently, Mishra *et al* (1986) described a monoclonal antibody generated to recognize the BSF-1 receptor. This monoclonal antibody, G48, mimics the biological effects of BSF-1 on small resting B cells (increase in Ia, co-stimulation with anti-Ig). Several groups have reported that monoclonal anti-Lyb 2.1 stimulates DNA synthesis in resting B cells (Subbarao and Mosier, 1983; Rabin *et al*, 1986a; Yakura *et al*, 1986). Low concentrations of anti-Lyb 2.1 co-stimulate with anti-IgM. Yakura *et al* (1986) have also observed that anti-Lyb 2.1 blocks absorption of BSF-1 by B cells from DBA/2 mice. These results suggest that anti-Lyb 2.1 may be directed to the BSF-1 receptor.

Another BCGF, BCGF-II, was identified as a T cell-derived product which synergized with $DXSO_4$ to induce proliferation of B cells (Swain *et al*, 1983). It also causes short-term proliferation of the *in vivo* BCL-1 tumor line. This factor was found in culture supernatants of the alloreactive T cell line C.C3.11.75. It can be distinguished from BSF-1 by phenyl sepharose chromatography and has an approximate molecular weight of 60 kD as determined by SDS-PAGE.

Another form of BCGF-II with distinct biochemical properties but almost identical activity, can be obtained from EL-4 culture supernatants induced by PMA [(EL-4)BCGF-II] (Dutton *et al*, 1984). This PMA-induced BCGF-II maintains proliferation of purified B lymphocyte blasts and induces their differentiation into Ig-secreting cells (Muller *et al*, 1985).

B151-TRF is found in constitutive supernatants produced by B151K12 T cell hybridoma (Takatsu *et al*, 1980a). It is defined as a factor that drives antigen-stimulated B cells into specific AFC development (Takatsu *et al*, 1980a,1980b), and anti-Ig-activated B cells into polyclonal Ig synthesis (Nakanishi *et al*, 1983). The same factor induces IgM secretion in BCL-1 cells (Takatsku *et al*, 1983). B151-TRF has an apparent molecular weight of 50 to 60 kD as determined by gel chromatography, and binds to N-acetyl galactosamine residues (GalNAc). Both growth and differentiation of B cells induced by B151-TRF were inhibited by the addition of GalNAc to the culture (Takatsu *et al*, 1985), indicating that binding of the GalNAc residue on B151-TRF to the TRF receptor plays a crucial role in the stimulation of B cells (reviewed in Hamaoka and Ono, 1986).

EL-TRF is found in PMA-induced supernatants of the cloned murine thymoma EL-4. Like B151-TRF, EL-TRF triggers antigen-activated B cells to generate specific AFC (Howard *et al*, 1982) and anti-Ig-stimulated B cells to generate Ig (Nakanishi *et al*, 1983). Kinetic analysis shows that EL-TRF acts at a much later stage of the antibody response than does B151-TRF. It does not act in either the anti-IgM or the $DXSO_4$ synergy proliferation assay, distinguishing it from the two proliferation factors BSF-1 and BCGF-II which are also found in induced EL-4 sup. Biochemical studies indicate that EL-TRF is trypsin sensitive and has an approximate molecular weight of 30-40 kD as determined by gel filtration analysis (Howard *et al*, 1984).

Two other kinds of B cell differentiation factors, BCDFμ and BCDFγ, were obtained from the Con A-stimulated T cell line PK7.1.1. (Isakson *et al*, 1982). BCDFμ induces BCL-1 cells or B cells to secrete IgM. An increase in cytoplasmic mRNA for the μ chain of the secreted form of IgM was observed after induction of BCL-1 with BCDFμ (Brooks *et al*, 1983). BCDFγ induces LPS-activated B cells to secrete IgG (Isakson *et al*, 1982). In the presence of both LPS and BCDFγ, a striking increase in γ_1-specific RNA is observed with a concomitant decrease in both γ_3- and γ_{2b}-specific mRNA. LPS alone induces secretion of small amounts of IgG_{2b}, IgG_3, and IgG_1. These results demonstrate that selective T cell-derived lymphokines can act on B cells to regulate levels of γ_1 and γ_3 (Jones *et al*, 1984,1985). It was not clear whether the IgG_1 induction factor (BCDFγ) induces selective activation of B cells which are already committed to IgG_1 production, or whether it directs the class switch to increase IgG_1-producing B cells. Characterization of this lymphokine by deoxynucleic acid (cDNA) cloning demonstrated that the product of the cloned BCDFγ induction factor gene was also able to induce an increase in Ia expression on B cells reported previously by Noelle *et al* (1984), and to synergize with anti-Ig in inducing DNA synthesis in the classical BSF-1 assay (Howard and Paul, 1983a). Recent studies based on biochemical similarities had also suggested that BSF-1 and BCDFγ may be the same lymphokine (Vitetta *et al*, 1985; Sideras *et al*, 1985). Therefore, the IgG_1 induction and BSF-1 activity are, indeed, functions of the same lymphokine.

Lee *et al* (1986) have also reported that a cloned BSF-1 gene product induces Ia expression on resting B cells and enhances IgG_1 and IgE production by B cells. The same factor stimulates T and mast cell lines. These results (Noma *et al*, 1986; Lee *et al*, 1986) demonstrate that a single cDNA clone encodes a protein which exhibits BSF-1 and BCDFγ activities, activates T and mast cell lines, and induces IgE secretion by B cells. These authors have jointly proposed that this lymphokine be designated "interleukin-4".

Recently, Yokota *et al* (1986) isolated a human cDNA clone homologous to mouse BSF-1. This gene product stimulated proliferation of human T_h cell clones and anti-IgM-activated human B cells.

The description of cloned gene products that exhibit multiple B cell regulatory and other activities previously ascribed to different lymphokines, suggests that a single lymphokine interacting with B cells in distinct differentiative states has different regulatory effects. Therefore, it may be an oversimplified view to consider the B cell's response as an ordered series of independent events, each of them regulated independently by the action of different controlling elements.

A number of studies have led to the hypothesis that B cells are not responsive to differentiation signals before receiving the signal(s) which initiate cell division (Kishimoto *et al*, 1984). This theory implies that receptors for lymphokines which induce differentiation are expressed only after the cells are committed to at least one round of division and are in late G_1. This hypothesis has been challenged by studies demonstrating that small resting B cells can be stimulated to polyclonal Ig secretion by T cell-derived supernatants without the addition of antigen, mitogen, or anti-Ig. Melchers and his associates described antigen-nonspecific helper factors from T_h cell lines, B cell replication and maturation factor (BRMF), that induces the replication and maturation of B cells (Melchers *et al*, 1980a,b). Their results indicate that BRMF acts on small resting B cells that have been activated in an MHC-restricted fashion by T_h and antigen, inducing them to progressively enlarge into blast-like cells and mature to secrete IgM with a total lack of proliferation. BRMF has not yet been compared to any other factor.

Sidman *et al* (1984c) also identified a BCDF acting on resting B cells and designated as B cell maturation factor (BMF); BMF are heterogenously-glycosylated multimeric glycoproteins with apparent molecular weights of 50 to 55 kD as determined by gel chromatography consisting of subunits of 16 kD by SDS-PAGE. B151-TRF and BMF have very similar features (Sidman *et al*, 1984c). BMF induces polyclonal Ig secretion of all subsets of normal B cells, including neonatal B cells, *nu/nu* B cells, and B cells from CBA/N mice (Sidman and Marshall, 1984a). Most, if not all, normal resting B cells in adult mouse spleen inducible by BMF are accompanied by a modest proliferation response. Mayer (1986) suggests that there is proliferation-dependent and proliferation-independent BCDF, and resting B cells may have full complement of receptors for BCDF as well as BCGF. In contrast, Simpson and Isakson (1986) showed that IgM secretion, in response to BCGF-II, requires DNA synthesis. Amphidicolin, used to prevent DNA synthesis in these experiments, blocks DNA polymerase I and does not alter the native DNA structure like other drugs such as mitomycin C. These data suggest that current models of B cell activation and maturation may require reassessment of the proliferative phase of B cell maturation.

Other Factors Involved in B Cell Activation

IFN-γ has been shown to either enhance or inhibit antibody production in polyclonal and antigen-specific immune responses (Friedman and Vogel, 1984). IFN-γ is a component of TRF (Zlotnik *et al*, 1983; Leibson *et al*, 1984), and the TRF activity present in supernatants of Con A- phytohaemmaglutinin (PHA)-, or mixed leukocyte reaction-stimulated cells is markedly diminished by antibodies to IFN-γ (Brunswick and Lake, 1985). This inhibition is reversed by the addition of recombinant IFN (r IFN)-γ. IFN-γ was reported to enhance *in vivo* murine B cell responses to soluble antigen (Nakamura *et al*, 1984), and to induce resting B cells and B cell lymphomas to secrete Ig (Sidman *et al*, 1984b). It also enhanced the *in vitro* antibody response of B cells to sheep red blood cells (SRBC) in the presence of IL-1 and IL-2 (Leibson *et al*, 1984). IFN-γ does not prevent an anti-Ig antibody-induced increase in B cell size. It does, however, suppress a BSF-1-induced increase in expression of Ia and preparation for DNA synthesis. Expression of other surface determinants was not altered by IFN-γ (Mond *et al*, 1985; Rabin *et al*, 1986b). Furthermore, IFN-γ effectively inhibits proliferation of soluble anti-Ig-stimulated B cells in the presence of BSF-1 but shows little suppressive activity on proliferation induced by sepharose-bound anti-Ig antibody (Mond *et al*, 1985). The inhibitory effects mediated by r IFN-γ on proliferation were achieved with as low as 0.001 U/ml. This is in contrast to amounts (10 U/ml) required for optimal induction of B cell differentiation by IFN (Sidman *et al*, 1984b; Murray *et al*, 1985). In summary, the role of IFN-γ in immune regulation may exhibit differential effects depending on the manner in which B cells are stimulated and the combination of lymphokines used.

IL-2 is a T cell-derived glycoprotein of 133 amino acids released by activated T lymphocytes and functions as a T cell growth factor (Robb *et al*, 1981) inducing proliferation of activated T cells *via* specific cell surface receptors (Smith, 1984). Until recently, it was generally accepted that resting or activated B cells do not respond directly to IL-2 but require other T cell-derived lymphokines for their proliferation. IL-2 did not show any effect on proliferation and differentiation of B cells (Puré *et al*, 1982). Neither IL-2 receptors (Robb *et al*, 1981) or antigens comparable to Tac (Osawa and Diamantstein, 1984) were found in LPS-stimulated murine B cells. Others, however, showed that depletion of IL-2 from co-factor-rich supernatants by absorption on IL-2-dependent T cell lines also removed a factor required for B cell differentiation (Leibson *et al*, 1981; Parker, 1982; Swain and Dutton, 1982). Recent studies have demonstrated the presence of a cell surface molecule which is recognized by monoclonal antibody directed against the IL-2 receptor, on murine B cell lymphomas (Korsmeyer *et al*, 1983), normal murine B lymphocytes activated with LPS (Malek *et al*, 1983), normal human B cells activated by PWM (Deeper *et al*, 1983), anti-μ antibody, PMA, and SAC, as well as on human B cell lines (Muraguchi *et al*, 1985). The presence of IL-2 receptors appears to correlate with the ability of IL-2 to induce proliferation and differentiation of B cells.

Immunoprecipitation analysis of surface-labelled blasts indicates that B and T cells have IL-2 receptors of similar relative molecular mass and that both B and T cells express high affinity (apparent dissociation constant, k_d ~20pM) and low affinity (k_d ~1,000pM) IL-2 receptors at a ratio of ~1:10 (Robb *et al*, 1984; Lowenthal *et al*, 1985). Antibodies to either IL-2 receptor (anti-Tac or monoclonal antibody PC61) inhibit both T and B cell function. B blasts, however, express half as many total IL-2 binding sites as T cell blasts (as estimated from PC61 binding).

In most studies, B cells required pre-activation with LPS (Malek *et al*, 1983), LPS and anti-Ig (Zubler *et al*, 1984), or SAC (Mingari *et al*, 1984; Nakagawa *et al*, 1985; Tsudo *et al*, 1985) to proliferate in response to IL-2. Similarly, IL-2-induced differentiation of B cells was shown to require B151-TRF (Nakanishi *et al*, 1984), BCDF (Mayer *et al*, 1985), IFN (Bich-Thuy *et al*, 1986), or SAC and IFN (Mingari *et al*, 1984; Jelinek and Lipsky, 1986). IL-2 alone, however, was reported to trigger differentiation of murine B cells in response to TI antigens (Pike *et al*, 1984; Striebich *et al*, 1986).

To summarize, IL-2 acts as a co-stimulator in inducing proliferation and differentiation of B cells in the presence of other factors. These findings suggest the existence of different pathways for B cell differentiation which require different combinations of T cell factors such as IL-2 and BCDF (Mayer *et al*, 1985), IL-2 and BCGF, or IL-2 and IFN-γ (Nakagawa *et al*, 1985; Bich-Thuy *et al*, 1986; Jelinek and Lipsky, 1986).

The growth and differentiation of hematopoietic cells is mediated by a number of glycoproteins, collectively known as CSF including granulocyte/macrophage CSF (GM-CSF or CSF-1) and multi-CSF (interleukin-3) (Metcalf, 1981; Dexter, 1984). Interleukin-3 is produced by activated T lymphocytes or T lymphomas (Ihle *et al*, 1982; Ihle, 1984). This multilineage hematopoietic growth regulator that initiates the proliferation and differentiation of multipotent stem cells including mouse B cell precursor. It has, however, been reported to have no effect on mature B lymphocytes (Ihle, 1984).

Role of Antigen-Presenting Cells in B Cell Activation

Although it has long been known that cellular collaboration in responses to TD antigens requires the presence of A cells, their precise role has never been well defined. TI antigens, divided into two groups, TI-1 and TI-2 (Mosier *et al*, 1977), differ in their requirement for A cells. TI-2 antigens are often macrophage-dependent, whereas TI-1 antigens, with the exception of LPS (Fernandez and Severinson, 1983), are less or not at all macrophage-dependent (reviewed in Mond, 1982). In short, B cell responses to TD antigens, TI-2, and LPS stimulation are A cell-dependent (reviewed by Corbel and Melchers, 1983). A cells from different sources such as peritoneal exudate cells, spleen cells, cells from the macrophage line P388D1, thymocytes, and macrophage products, were shown to reconstitute such B cell responses (Hoffman, 1980). Later findings identified IL-1 as the maturation-inducing activity in macrophage supernatants (Howard *et al*, 1983b). Using several methods to stimulate B cell responses, a number of laboratories have now concluded that IL-1 acts in synergy with other lymphokines in promoting B cell proliferation and differentiation (discussed next).

Role of IL-1 in B Cell Activation

IL-1 was originally thought to be a factor solely produced by cells of monocyte-macrophage lineage, that induces secretion of T cell growth factor from T cells. Later, many cell types, including endothelial cells, large granular lymphocytes, and Epstein Barr Virus-transformed B cells, were found to secrete IL-1 and a large number of biological activities were attributed to IL-1. Recently, T cell clones that produce an IL-1-like activity after stimulation by antigen-presenting B cells have been reported (Tartakovsky *et al*, 1986). IL-1-like activities have been ascribed to proteins with isoelectric points (pI) of 5.0 and 7.0. LPS-stimulated murine P388D1 macrophage line produces predominantly pI 5.0 IL-1. The gene encoding pI 5.0 IL-1 has been cloned by Lomedico *et al* (1984). Human peripheral blood monocytes and monocyte lines such as TMP-1, produce both pI 7.0 and pI 5.0 IL-1 at a ratio of 9:1 (for review, see Oppenheim *et al*, 1986). March *et al* (1985) have identified two distinct human IL-1 genes encoding IL-1α (pI 5.0) and IL-1β (pI 7.0).

The importance of macrophages and macrophage-secreted products in the maturation of B cells led to studies implicating IL-1 in proliferation and differentiation of B cells (Hoffman, 1980; Kincade *et al*, 1982; Howard *et al*, 1983b; Oppenheim *et al*, 1986). B lymphocytes are reactive to IL-1 during at least two stages of development; first, at the B cell precursor stage and, second, following antigen stimulation. IL-1-driven maturation of a cell line with pre-B cell characteristics (cytoplasmic μ chain without L chain) leads to κ L chain production and subsequent membrane-Ig expression in this pre-B cell line (Giri *et al*, 1984). The second stage at which IL-1 has been implicated is during activation of B lymphocytes (Liebson *et al*, 1982; Howard *et al*, 1983b; Booth and Watson, 1984; Corbel and Melchers, 1984; Melchers and Lernhardt, 1985). Howard *et al* (1983b) demonstrated that IL-1 is an activating lymphokine and could enhance the proliferative response of B cells activated with anti-IgM to BSF-1. Kinetic analysis investigating the roles of BSF-1 and IL-1 in the B cell cycle suggested that BSF-1 operates on G_0 or early G_1 cells and IL-1 acts at some later part of G_1. Later, Melchers and Lernhardt (1985) investigated the role of α type BCGF produced by the P388D1 macrophage line, on murine B cells synchronized after LPS activation. Restimulation of cells with anti-μ indicated the requirement for α-factors 3-4 hr before the cells enter S phase.

Booth and Watson (1984) showed that a B cell growth and differentiation factor (BGDF/IL-1) derived from the myelomonocytic line WEH1-3 and inseparable from IL-1, synergizes directly with anti-IgM to induce B cell proliferation and maturation to antibody secretion. Lipsky *et al* (1983) reported that the antibody response of peripheral blood lymphocytes to PWM could be inhibited by antibody specific for IL-1. Other studies showed that IL-1 failed to induce differentiation of activated B cells and was not required for induction of Ig secretion by BCDF (Liebson *et al*, 1982; Falkoff, 1984). However, it was found to synergize with BCDF in inducing Ig secretion. Recently, recombinant IL-1 (r IL-1) was reported to be sufficient for both growth and maturation of antigen-specific B cells (Pike and Nossal, 1985). An additive effect was observed when used in combination with recombinant IL-2 (r IL-2). Shirakawa *et al* (1986) found that macrophages or IL-1 are required for the induction of IL-2 receptors on human B cells.

There has been considerable doubt whether the observed effects of IL-1 were due to direct effects on the B cell itself or indirect effects mediated through contaminating A cells (Howard *et al*, 1983b). Some reports suggest that the production of IL-1 by normal human and murine B lymphocytes upon activation may account for the variable effects of exogenously added IL-1 (Kurt-Jones *et al*, 1985; Matsushima *et al*, 1985; Pistoia *et al*, 1986). This B lymphocyte-derived IL-1 activity is biochemically and immunologically similar to monocyte-derived IL-1.

D. Molecular Mechanisms of B Cell Activation

A number of biological systems including the lymphoid system have been used to study the signals that mediate cell growth and differentiation. Cell surface receptors receive external stimuli which are transduced *via* "second messengers" to intracellular sites to control proliferation, secretion, and growth. Some of these second messengers such as cyclic adenosine monophosphate (cAMP), cyclic guanosine monophosphate (cGMP), Ca^{2+}, K^{2+}, inositol phosphates, and diacylglycerol (DAG), have been extensively studied. In B lymphocytes, signals generated after ligand interaction with surface Ig receptors in concert with signals derived from soluble factors trigger B cell growth and differentiation (Howard and Paul, 1983a; Vitetta *et al*, 1984). Studies on this subject suggest the existence of a complex series of transducing molecules and second messengers similar to that of other cell systems.

Activation of Na^+/K^+ Adenosine Triphosphatase (ATPase)

Cross-linking of surface structures by mitogenic ligands induces early, membrane-associated phenomena prior to the entry of resting lymphocytes into the cell cycle. Some of these early phenomena include changes in membrane transport of Na^+, K^+, and Ca^{2+}

(Quastel and Kaplan, 1970; Segel and Lichtman, 1979; Segel *et al*, 1983). Na^+/K^+ ATPase catalyzes a coupled transmembrane exchange of three internal Na^+ ions for two external K^+ ions (Sweadner and Goldin, 1975) driven by the free energy of hydrolysis of one molecule of adenosine triphosphate. However, it is not clear that this activation is sufficient or even essential for subsequent cell proliferation. Increased Na^+/K^+ ATPase activity has been reported in PHA- (Quastel and Kaplan, 1970; Segel and Lichtman, 1979) or Con A- (Avendunk, 1976) stimulated human peripheral blood lymphocytes, anti-Ig-stimulated human neoplastic B cell populations (Heikkila *et al*, 1981) and LPS-stimulated mouse spleen cells (Owens and Kaplan, 1980). Ouabain, a specific inhibitor of this enzyme (Schwartz *et al*, 1975), can inhibit mitogen-induced stimulation of lymphocytes (Quastel and Kaplan, 1968,1970; Avendunk, 1976). These results implicate Na^+/K^+ ATPase in the initiation of lymphocyte activation.

An increase in internal Na^+ was postulated as a possible mechanism for the Na^+/K^+ pump activation (Kaplan and Owens, 1980). Indeed, an increase in intracellular Na^+ concentration was observed following PHA stimulation of human peripheral blood lymphocytes (Segel and Lichtman, 1979; Avendunk and Gunther, 1980). Similar increases in the Na^+/K^+ pump activity were reported to occur after growth stimulation of fibroblasts (Smith and Rozengurt, 1978) and neuroblastoma cells (Molenaar *et al*, 1981). They also appeared to depend on an increased Na^+ influx which could be inhibited by amiloride. Likewise, the increased $^{86}Rb^+$ (K^+ analogue) influx observed upon anti-Ig stimulation of B lymphocytes, was inhibited with amiloride (Rosoff and Cantley, 1983). In another model, the differentiation of the murine splenic tumor cell line 70Z/3, induced by LPS, increased intracellular Na^+ concentration which could be blocked by amiloride (Rosoff and Cantley, 1983). Raising the $[Na^+]$ with ouabain or Na^+ ionophore, monensin causes expression of surface IgM almost as effectively as LPS. These data suggest that the activation of the Na^+/K^+ pump depends on an increased Na^+ influx.

Using human neoplastic B cell populations, Heikkila *et al* (1981) demonstrated an increased influx of $^{86}Rb^+$ upon activation with anti-Ig. Subsequent studies have suggested that the $^{86}Rb^+$ response probably requires mobilization of intracellular Ca^{2+} (Heikkila *et al*,

1985) but the increased $[Ca^{2+}]_i$ was shown to be insufficient for a $^{86}Rb^+$ response.

Activation of Amino Acid Transport

Several transport systems for amino acids have been characterized in mammalian cells (Christensen, 1973; Guidotti *et al*, 1978). The A system transports neutral amino acids with short, polar, or linear side chains and the transport is dependent on a Na^+ gradient across the plasma membrane. The ASC system, so named because it transports alanine, serine and cysteine, is also Na^+-dependent. The L system transports amino acids such as leucine and phenlyalanine which have branches or circular side chains and is insensitive to the Na^+ gradient. Amino acid depletion results in an increase only in the A system amino acid uptake in lymphocytes (Frengley *et al*, 1974; Segel and Lichtman, 1981) and other cell types. Human lymphocytes stimulated to enter the cell cycle by lectin treatment cause an increase in the A, ASC, and L transport systems (Mendelsonn *et al*, 1971; Avendunk, 1972; Whitney and Sutherland, 1973; Segel and Lichtman, 1981). While a two-fold increase in amino acid uptake by the A or ASC system is observed after 4 hr of lectin treatment, a similar increase in amino acid uptake by the L system was shown to occur after 16-20 hr of stimulation (Segel and Lichtman, 1981). The pathway of amino acid transport in T or B lymphocytes may be different, and definitive studies characterizing amino acid transport systems are lacking (Segel *et al*, 1983).

Ca^{2+} Mobilization, Phosphoinositol Turnover, and Protein Kinase C

In many cell types, binding of ligand to receptor enhances the turnover of membrane phosphoinositides (inositol phospholipids), which is accompanied by an increase in the intracellular concentration of Ca^{2+} (Michell, 1975). Later studies indicated that the breakdown of phosphatidylinositol bisphosphate by phospholipase C to DAG and inositol triphosphate (IP_3) is probably the initial event (Berridge, 1984). DAG is phosphorylated by DAG kinase to phosphatidic acid, which is then recycled back to phosphoinositide. Phosphoinositide degradation and Ca^{2+} mobilization appear to be fundamental mechanisms in the transduction of a wide variety of extracellular stimuli into cellular responses, including cell proliferation. Fisher and Mueller (1968) first linked enhanced phosphoinositide turnover with lymphocyte activation. Initial evidence for mitogen-stimulated phosphoinositide turnover in B lymphocytes was reported by Maino *et al* (1975). Recently, direct monitoring of degradation products from prelabelled phosphoinositide (Bijsterbosch *et al*, 1985) confirmed the earlier demonstration of enhanced phosphoinositide turnover in anti-Ig-stimulated murine B lymphocytes (Coggeshall and Cambier, 1984) but not in LPS- or PMA-stimulated cells (Betel *et al*, 1974).

Post-translational phosphorylation of serine and threonine residues in specific cellular proteins plays an important role in mediating the action of various hormones and growth factors. The change in lipid structure during cell activation could, in the presence of Ca^{2+}, enhance the binding of membrane phospholipid to PKC and thereby promote changes in membrane potential. Both primary products of phosphoinositide degradation, IP_3 and DAG, have been postulated to act as second messengers in invoking cellular responses (Berridge, 1984). While IP_3 is thought to be involved in Ca^{2+} mobilization from intracellular stores in B lymphocytes (1,4), DAG has been demonstrated to be an essential co-factor of PKC (Kishimoto *et al*, 1980; Ku *et al*, 1981; Ransom and Cambier, 1986a).

In general, phosphoinositide degradation is associated with an increase in Ca^{2+} mobilization (Michell, 1975; Bijsterbosch *et al*, 1985) and this Ca^{2+} mobilization plays an important role in lymphocyte activation (Whitney and Sutherland, 1973). Other studies showed a requirement for external Ca^{2+} (Desaymard *et al*, 1980), enhanced Ca^{2+} influx (Freedman, 1979), and release of Ca^{2+} from internal stores (Braun *et al*, 1979) upon stimulation of B cells. Using the fluorescent Ca^{2+} indicator quin-2, an increase in $[Ca^{2+}]_i$ was observed in anti-Ig-stimulated B cells (Pozzan *et al*, 1982; Bijsterbosch *et al*, 1985; Ransom *et al*, 1986b). However, LPS and PMA failed to provoke a significant increase in $[Ca^{2+}]_i$ (Bijsterbosch *et al*, 1985). While there is general agreement that the concentration of Ca^{2+} in the cytosol influences mitogenic stimulation, the relative dependence of the cellular function on extracellular Ca^{2+} or Ca^{2+} released from intracellular stores is debatable. Membrane depolarization and increase in surface Ia expression can be induced by increasing $[Ca^{2+}]_i$ with the Ca^{2+} ionophores A23187 and ionomycin (Ransom and Cambier, 1986a; Dennis *et al*, 1986). Ethyleneglycol-bis-(β-aminoethyl ether)-N,N,N',N'-tetra acetic acid (EGTA) inhibits the Ca^{2+} ionophore-induced increase in Ia expression and partially diminishes the surface Ia augmentation resulting from anti-Ig antibody but has no effect on LPS-stimulated Ia expression. On the other hand, altering the Ca^{2+} availability by EGTA inhibits thymidine uptake stimulated by LPS or anti-Ig (Dennis *et al* 1986). These findings suggest that extracellular Ca^{2+} may be required for later events in B cell activation, while earlier events may be primarily dependent upon intracellular Ca^{2+} stores.

Recently, Proust *et al* (1986) confirmed the earlier suggestions that activation signals (Ca^{2+}, PKC) leading to different lymphokine-dependent proliferation may proceed *via* different pathways. BSF-1-dependent B cell proliferation required co-stimulation with anti-μ or Ca^{2+} ionophore (ionomycin), both of which induced an increase in $[Ca^{2+}]_i$ without induction of PKC. On the other hand, IL-2-dependent B cell proliferation was reported to require pre-activation with both anti-μ and LPS. The LPS-derived signal could be replaced by PMA, adding weight to the previously suggested possibility (Wightman and Raetz, 1984) that LPS and PMA activate PKC directly without provoking a significant increase in $[Ca^{2+}]_i$ (Bijsterbosch *et al*, 1986). Therefore, both an increase in $[Ca^{2+}]_i$ and the activation of PKC are required for IL-2-induced proliferation. A search for specific phosphorylated cellular proteins implicated in mediating mitogenesis is now in progress in various laboratories. To this effect, Justement and Cambier (1986) have reported selective phosphorylation of a 44 kD protein upon addition of BSF-1 to plasma membranes. LPS or PMA had no effect on phosphorylation of the 44 kD band. These results suggest that the activation of a cAMP- or Ca^{2+}-dependent protein kinase may be associated with binding of BSF-1 to the plasma membrane.

Regulation of B Cell Response by Prostaglandins

Prostaglandin (PG) production has been demonstrated primarily for macrophages and to a lesser extent for lymphocytes (Tomar *et al*, 1981). The effects of PG on B cell activation and differentiation have not been clearly defined. Protein A-, SAC-, and PHA-stimulated growth of B cell colonies from human peripheral blood (Whisler and

Newhouse, 1982) are significantly inhibited by the physiologic concentration of prostaglandin E_2 (PGE_2). *In vivo* studies in mice show that the antibody response could be significantly enhanced by cyclooxygenase inhibitors (Webb and Osherhoff, 1976) such as indomethacin. PGE_2 is one of the major products of the cyclooxygenase pathway of arachidonic acid. However, Morito *et al* (1980) found inhibition of cyclooxygenase activity to have no effect on PWM-induced Ig secretion, whereas large concentrations of PGE_2 were reported to be inhibitory. By contrast, Staite and Panayi (1982) reported that cyclooxygenase inhibition with indomethacin inhibits PWM-stimulated Ig secretion and low concentration of PGE_2 could reverse this inhibition. Overall, the data support the conclusion that PGE_2 may play an important role in regulating the magnitude of B cell responses, although the details of its mechanism of action are still obscure.

Role of Cyclic Nucleotides

The possibility that the cyclic nucleotides, cAMP, and cGMP, may regulate lymphocyte function has been reviewed extensively (Hadden and Coffey, 1982; Coffey and Hadden, 1984). Increased levels of intracellular cAMP appear to be associated with the inhibition of *in vitro* mitogen responses (Parker *et al*, 1974; Strom *et al*, 1977; Novogrodsky *et al*, 1979; Hadden and Coffey, 1982). To summarize the role of cAMP, it can be stated that cAMP inhibits the induction of proliferation of both T and B cells and inhibits the production of lymphokines. The only events promoted by cAMP to date involve differentiative steps and Ig receptor capping (Butman *et al*, 1981). Recently, Gilbert and Hoffman (1985) showed that cAMP and IL-1 stimulated antigen-specific and polyclonal antibody responses when added together to cultures of highly purified B cells. They proposed that IL-1 and an elevation of cytoplasmic cAMP represent minimal signal requirements for B cell activation. In contrast, agents that increase cGMP levels promote or augment all cellular functions that are inhibited by cAMP (Strom *et al*, 1977; Hadden and Coffey, 1982). Specific mitogens cause early increases in cGMP in B cells (Watson, 1975) as well as T cells (Hadden *et al*, 1972; Coffey *et al*, 1981). Mouse spleen cells stimulated with Con A (Wang *et al*, 1978), human lymphocytes triggered with PHA (Carpentieri *et al*, 1980) or Con A, PMA, and Ca^{2+}

ionophore (A23187) (Rochette-Egly and Kempf, 1981) undergo an early rise in cAMP, followed shortly by an increase in cGMP-dependent protein kinase and a later increase in cAMP and its kinase.

The role of cAMP or cGMP in cell triggering and the mechanism by which these cyclic nucleotides are elevated is still unclear. In spite of heterogeneity in receptor-mediated triggering mechanisms, most tissues possess two classes of receptors for controlling cellular function and proliferation. One class triggers the production of cAMP while the other induces inositol phopholipid turnover, Ca^{2+} mobilization, and cGMP production (Nishizuka, 1984). Surprisingly, cGMP-dependent and cAMP-dependent protein kinases show very similar catalytic properties. Several lines of evidence suggest that cGMP is a negative, rather than a positive, messenger and provides an immediate feedback control to prevent excessive response (Haslam, 1980; Takai *et al*, 1981). In those cells where cAMP generated by "negative signals" inhibits the transmission of 'positive signals' (i.e., PI breakdown, Ca^{2+} mobilization), cAMP and cGMP do not antagonize each other but block the receptor-linked degradation of PI in membranes and thereby inhibit cellular functions and proliferation by counteracting the activation of PKC. These inhibitory actions of cyclic nucleotides are probably mediated through effects on the mobilization of Ca^{2+} *via* the activation of protein kinases A and G (reviewed by Nishizuka, 1984).

Role of G Proteins

Interaction of regulatory molecules (hormones, neurotransmitters, etc.) with appropriate ligands on the cell surface result in stimulation or inhibition of adenylate cyclase activity. This results in subsequent alterations of intracellular phosphorylation as a consequence of the actions of cAMP-dependent protein kinases (for example, protein kinase A). Both stimulatory and inhibitory receptors communicate with a pair of homologous guanine-nucleotide-binding regulatory proteins — one, G_s, mediates stimulation of adenylate cyclase activity while the other, G_i, is responsible for inhibition (reviewed by Gilman, 1984). The G proteins are composed of two subunits, α and β; the β subunit is common to both proteins while the larger, α, subunit of each protein

($G_s\alpha$ and $G_i\alpha$) is different. $G_s\alpha$ is sufficient to activate adenylate cyclase and the β subunit of G_s inhibits this activation. The inhibitory activity of the β subunit of G_i is exerted at the level of the stimulatory coupling protein. $G_i\alpha$ plays the role of an anti-inhibitor of adenylate cyclase activity. This polypeptide may thus be an excellent candidate as a mediator of other functions such as Ca^{2+} gating and phosphatidylinositol metabolism. To this effect, a phosphodiesterase (phospholipase C), that stimulates the breakdown of the byproduct of phosphatidylinositol into IP_3 and DAG, may be controlled by coupling of receptors for regulatory molecules to the enzyme through a guanosine triphosphate-binding protein as in the mechanism effecting the interaction between receptors and adenylate cyclase (Gomperts, 1983; Gilman, 1984). However, the underlying mechanisms of the phenomenon outlined above need yet to be confirmed for activation of B cells.

Upon reviewing the literature, it appears that no consensus has emerged concerning the identity of the cytokines believed to control proliferation and antibody synthesis in B cells. Contradictory statements concerning the requirement for cytokines in B cell activation are possibly due to the different experimental models used by different investigators, many of which may have been insufficiently standardized with respect to B cell heterogeneity, contamination of B cells with other cell types, and definition of cytokines. The work described in this thesis was undertaken to clearly identify the cytokines required in B cell activation using an experimental model in which B cells specific for antigen (CRBC) were enriched to a degree at which T cells and macrophages were limiting.

II. Materials and Methods

Animals. B10.BR/SgSn/J//Chl mice were obtained from Ellerslie Animal Farm, University of Alberta. Spleen cells from male or female animals aged 8-12 weeks were used for rosetting B cells, and those aged 4-6 weeks were used as a source of thymocytes when necessary.

Antigen. Chicken lines homozygous for the B^2 MHC haplotype were bred by the Biosciences Animal Services, University of Alberta, and have been described in detail previously (Shiozawa *et al*, 1980). CRBC were stored at 4 °C for up to 1 week in Alsever's solution. Before use, CRBC were centrifuged, depleted of buffy-coat cells, washed twice by centrifugation for 10 min at 2000 *g*, and resuspended in Dulbecco's phosphate-buffered solution (PBS) containing 5% fetal calf serum (FCS, Grand Island Biological Company, Grand Island, NY).

Mitogen. LPS was a phenol extract of *E. coli* serotype 055.B5 (Sigma Chemical Co., St. Louis, MO). A 1 mg/ml solution was prepared in normal saline, filtered through 0.45 µm filter (Nalgene, Sybron Corp., Rochester, NY), aliquoted and frozen until use. The LPS was used at a final concentration of 10 µg/ml of culture medium.

Recombinant IL-1. Both human (Gubler *et al*, 1986) and mouse (Lomedico *et al*, 1984) r IL-1 alpha were kindly provided by Dr. P. Lomedico (Hoffman-La Roche, Nutley, NJ). Human IL-1 (Lot 14993-74/ 102) had a specific activity of 10^8 units/mg, protein concentration of 20 µg/ml and an activity of 3 x 10^6 units/ml. It was stored in 25 mM Tris-Cl, 0.6 M NaCl (pH 8.1) at -20 °C. Endotoxin contamination was 20 units/ml. Mouse r IL-1 (Lot 14430-153) had a specific activity of 1 x 10^6 units/ml and was stored in 5 mM guanidine HCl at -20 °C. This preparation was also reported to be contaminated with trace amounts of endotoxin. The concentration of r IL-1 is expressed as units/ml of IL-1 activity as determined by suppliers.

Recombinant IL-2. Human r IL-2 (Lots G.8.61 and G.8.73) was prepared and kindly provided by Dr. C. Bleackley, Department of Biochemistry. The concentration of r IL-2 is expressed as units/ml of IL-2 activity as determined by Bleackley *et al* (1985).

Inhibitors. 10 mM ouabain (Sigma) was prepared by dissolving in warm saline and stirring for 15-20 min. Ouabain was stored at 4 °C until use.

EL-4 Supernatant. EL-4 (Farrar *et al*, 1982) was maintained in Roswell Park Memorial Institute (RPMI) 1640 medium (GIBCO) containing 7% horse serum (GIBCO), 50 mM β-mercaptoethanol (β-ME, Eastman Kodak Co., Rochester, NY), and 0.5% penicillin-streptomycin (GIBCO). For induction, EL-4 cells were washed, resuspended at a density of 1 x 10^6 cells/ml, and stimulated with 10 ng/ml of PMA (Sigma) for 24 hr. Culture supernatants were harvested by centrifugation for 20 min at 500 *g*. Ammonium sulfate (Fisher Scientific Co., Ltd., Fair Lawn, NJ) was added to 80% saturation (560 g/l) and stirred at 4 °C overnight. The precipitate was collected by centrifugation at 20,000 *g* for 1 hr, resuspended in a small volume of 0.01 M N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) buffer (pH 7.4), and dialyzed against several changes of 0.01 M HEPES containing 0.04 M NaCl (pH 7.4). After dialysis, the supernatant was sterilized by filtration (0.22 μ filter, Nalgene), aliquoted, and stored frozen. EL-4 sup was tested for its ability to induce proliferation as measured by ^{3}H-thymidine incorporation and differentiation of B cells as measured by hemolytic plaque assay. The dilution of EL-4 sup which was minimally active in inducing proliferation but optimally active in inducing differentiation of B lymphocytes, was determined for each batch of EL-4 sup. These fractions could be stored for a year without loss of their potential to trigger proliferation or differentiation. However, the ability to induce activation of Na^+/K^+ ATPase was lost if the EL-4 sup was stored for longer than 2 weeks.

Preparation of Affinity-Enriched B Cells. Rosette-forming cells (RFC) were prepared by a modification of the method of McConnell *et al* (1969). Mice were killed by cervical dislocation and their spleens excised and squeezed through a wire screen using a plastic syringe plunger into PBS containing 5% FCS. Cellular debri was removed by a quick spin, i.e., stopping centrifuge when it reached 300 *g*. The supernatant was transferred to a fresh tube and centrifuged for 7.5 min at 500 *g*. The washing was repeated, the cell pellet suspended in 2.5 ml PBS + 5% FCS/spleen, and 10 ml of cell suspension layered slowly over 10 ml Ficoll. Ficoll used was a solution of 10.98% Ficoll 400 (Pharmacia Fine Chemicals, Uppsala, Sweden) containing 29.5% sodium metrozoate (Sigma) in double-distilled water (DDW). This Ficoll solution had a density of 1.09 g/cm^3 and an osmolarity of 280-308 mOsm. Cells were centrifuged at 2000 *g* for 25 min at 20 °C. After centrifugation, the interphase contained the viable cells while the majority of red cells and dead cells were present in the pellet. The interphase cells were resuspended in PBS + 5% FCS and centrifuged at 500 *g* for 7.5 min. Two subsequent washes were done at 500 *g* for 7.5 min. The cells were counted in eosin (0.1% w/v, Fisher) and rosetted with CRBC. For rosetting, 2 x 10^8 cells/0.1 ml of 8% CRBC were used. The spleen cell pellet was gently suspended in 8% CRBC and left at 4 °C for 25 hr or overnight. After rosetting, the cells were resuspended in PBS + 5% FCS (3 ml/spleen), layered over Ficoll and centrifuged at 2000 *g* for 25 min at 20 °C to separate the rosetted cells (pellet) from the nonrosetted cells (interphase). The pellet was resuspended in a small volume of PBS + 5% FCS, transferred to a fresh tube, washed 3 times with PBS + 5% FCS, and the rosettes counted. The ratio of rosetted cells to contaminant nonrosetted cells varied from experiment to experiment. Less contamination of nonrosetted cells in the RFC preparation could be obtained by decreasing the time of the final Ficoll separation. A preparation of RFC obtained by decreasing the time of final Ficoll centrifugation to 4 min (RFC 4') was also used. Under these conditions, the ratio of rosetted cells to contaminant nonrosetted cells was less than 20:1. Differences in responses by RFC 25' and RFC 4' are discussed in the *Results* section.

Antigen (CRBC) was not removed from the RFC preparation before culture. Over 99% of these RFC were surface Ig-positive as determined by immunofluorescence.

Depletion of T Cells. Spleen cells were prepared (Shaw *et al*, 1981) in Leibovitz medium containing 50 mM β-ME and 0.1% gelatin. The cells were layered over Ficoll to remove dead cells and red cells as described above. Interphase cells were collected after centrifugation and washed with Leibovitz medium at 500 *g* for 7.5 min. The cell pellet was resuspended in an appropriate dilution of anti-mouse Thy-1.2 antibody (ascites fluid, New England Nuclear, Boston, MA) in Leibovitz medium (2 x 10^7 cells/ml of diluted anti-Thy-1.2). The activity of each batch of anti-mouse Thy-1.2 was evaluated by its ability to kill mouse thymocytes in the presence of complement. The antibody was diluted 1:100 in

Leibovitz medium and stored frozen at -70 °C. After 45 min on ice, rabbit complement (Low-Tox, Cedarlane Laboratories Limited, Hornby, Ontario, Canada) was added to the cell suspension at a final dilution of 1:10 and the cells incubated at 37 °C for 45 min. The cells were then pelleted and washed twice with Leibovitz medium. The viable cell recovery was determined by the eosin-dye exclusion method. The cells were layered over Ficoll to remove dead cells and used for culture after washing twice. The treatment of mouse splenocytes with anti-mouse Thy-1.2 and complement abolished the 4 day mitogenic *in vitro* response to Con A, whereas the response to LPS was only minimally affected. Fifty to sixty percent of the cells were recovered after treatment with anti-Thy-1.2 and complement.

Depletion of Macrophages. Macrophages were removed by adherence to plastic. Spleen cells (10-20 x 10^6/ml) were added to polystyrene dishes (100 x 20 mm, Falcon 3003, Becton Dickinson and Company, Oxnard, CA) in a total volume of 5 ml of RPMI supplemented with 10% FCS and 50 mM β-ME. After 1 hr at 37 °C, nonadherent cells were collected by gently swirling and decanting the medium. The nonadherent cells were collected, added to fresh polystyrene dishes, and the procedure repeated thrice. The remaining nonadherent cells were collected, washed, and used for preparation of RFC. On an average 30-35 percent of the input cells were recovered after adherent cell depletion.

Cell Cultures. For measurement of $^{86}Rb^+$ influx and proline uptake 6 x 10^4 RFC, or 2 x 10^6 spleen cells, or 2 x 10^6 anti-Thy-1.2-depleted spleen cells per ml of Click's medium (Click *et al*, 1972), supplemented with 5% FCS, 50 mM β-ME, and 0.5% penicillin-streptomycin (GIBCO), were cultured in 100 x 20 mm tissue culture dishes (Falcon). CRBC were added to cultures requiring antigen (0.1 ml of 8% CRBC suspension per 10^8 cells). Cells were stimulated with 10 μg/ml LPS or 50 μl/ml of EL-4 sup diluted to 1:40 or a combination of LPS and EL-4 sup. For assessment of proliferative and differentiative activity, 3 x 10^3 RFC, 6 x 10^3 RFC 4', 2 x 10^4 T cell-depleted spleen cells, or 2 x 10^5 spleen cells were cultured in 200 μl of medium in flat-bottom, 96-well microtiter plates (Linbro Scientific Co., Hamden, CT) supplemented with appropriate stimulatory factors. Cultures were incubated in a humidified atmosphere of 7% CO_2 in air at 37 °C.

^{86}Rb^{+} Influx Measurements. After 14 hr of culture, RFC were spun on Ficoll to remove the CRBC which by that time had become detached from the RFC, while spleen cells or spleen cells depleted of T cells were washed with PBS twice. After washing, the cells were resuspended in 0.75 ml of Tris-buffer (pH 7.4, Tris 20 mM, NaCl 121.3 mM, KCl 4 mM, $CaCl_2$ 1.8 mM, $MgSO_4$ 1 mM, KH_2PO_4 1 mM, and 5 mM glucose) containing FCS (GIBCO) and incubated at 37 °C for 10 min. Fifty μl of ^{86}RbCl (20 μCi/ml, NEN) diluted in Tris-buffer were added as a substitute tracer for K^+ at 37 °C to give a final specific activity of 5 μCi/ml of cell suspension. At various times after addition of ^{86}RbCl, 100 μl aliquots (2×10^5 cells) were transferred to 0.4 ml microfuge tubes containing 70 μl of silicon oil (Dow-Corning MI) (12 parts oil 550:13 parts oil 556) and layered over 20 μl of 2 M NaOH. The ^{86}Rb^{+} uptake was terminated by addition of 150 μl of ice-cold Tris-buffer without glucose and FCS and the cells pelleted by centrifugation in an Eppendorf Microfuge for 2 min. The tubes were frozen on dry ice, cut across the oil layer, and the tube tips transferred to scintillation vials containing 400 μl of 1% Triton X-100 (to dissolve the pellet) in 4 ml of scintillation fluid (ACS, Amersham Corporation). Samples were tested for radioactivity in a Beckman Scintillation Counter. To calculate the specific activity of the radioactivity in 5 μl of the supernatant after centrifugation, sample Eppendorf tubes were transferred to scintillation vials and counted as described above. Ouabain-insensitive ^{86}Rb^{+} influx was measured in the presence of 2 mM ouabain included in the medium. RFC were spun on Ficoll as usual to remove CRBC and washed in cold PBS. No significant loss of ^{86}Rb^{+} occurred from the cells during the washing. Samples were taken for measurement as described for influx.

The rate of ^{86}Rb^{+} uptake expressed as pmol/10^6 cells/min was calculated as follows:

1. SA (cpm/pmol) = cpm/ml + Z,

   where SA is the specific activity and Z is the concentration of K^+ in the medium (5 mM).

2. Rate of ^{86}Rb^{+} uptake pmol/10^6 cells/min) = X + SA + Y + 20,

   where X = cpm at 21' - cpm at 1'

   and Y = cells per sample (in millions)

$$\text{Std. deviation of rate} = \sqrt{\frac{SD_1^2}{n_1} + \frac{SD_{21}^2}{n_{21}}} \div SA \div Y \div 20$$

Measurement of Proline Uptake. Cells were prepared as described earlier and preincubated at 37 °C for 15 min in Tris-buffer containing 5% dialyzed FCS (GIBCO) to deplete the internal amino acid pool. Cells were resuspended in 0.4 ml Tris-buffer containing 5% dialyzed FCS and amino acid uptake was measured at 37 °C. Aliquots of 50 μl (2×10^5 cells) were transferred to 0.4 ml microfuge tubes containing 50 μl of Tris-buffer supplemented with 10 μCi/ml ^{3}H-proline (20 mCi/ml, NEN) and 100 μM proline (Sigma) layered over silicon oil and NaOH. At various times, the amino acid uptake was terminated as described for K^+ uptake. Counts trapped in extracellular space as measured by a duplicate assay at 4 °C, were subtracted. The remaining procedure was similar to that used for K^+ influx measurements including controls and estimation of specific activity. Na^+-independent proline uptake was measured by replacing Na^+ with choline in Tris-buffer.

The rate of proline uptake expressed in pmol/10^6 cells/min was calculated as follows:

1. SA (cpm/pmol) = cpm/ml ÷ Z,
   where Z is the concentration of proline in medium (100 μM).
2. Rate of proline uptake (pmol/10^6/min) = X' ÷ SA ÷ Y' ÷ 5,
   where X' = cpm at 5' - cpm at zero time
   and Y' = cells per sample (in millions)

$$\text{Std. deviation of rate} = \sqrt{\frac{SD_0^2}{n_0} + \frac{SD_5^2}{n_5}} \div SA \div Y \div 5$$

BSF-1 Assay. BSF-1 activity was assayed by a modification of the previously reported anti-IgM co-stimulator method (Howard *et al*, 1982). B cells were prepared from the spleens of 8- to 12-wk-old mice. The cells were depleted of T cells as described earlier. The resulting cells were layered over Ficoll to remove dead cells and cultured at 5×10^4 cells in 0.2 ml of RPMI/culture well in the presence of a two-fold dilution of 2.5 μg/ml affinity-purified F(ab')$_2$ fragment of goat anti-mouse IgM antibody (Rockland, PA) with or without EL-4 sup for 3 days. The cells were pulsed with ^{3}H-thymidine for 5-8 hr and harvested as discussed.

IL-2 Assay. The IL-2 assay was performed as described by Gillis *et al* (1978). Serial two-fold dilutions of the rat polyclonal IL-2 (rat T cell polyclone, Biomedical Products Division, Collaborative Research Inc., Mass 01273) and EL-4 sup were made, in triplicate, in 0.1 ml RPMI contained in flat-bottomed microtiter plates. Fifty thousand HT-2 cells in 0.1 ml of medium were added to each well. The plates were incubated for 24 hr and cells pulsed with 2 μCi of methyl [^{3}H-thymidine (2 μCi/Mmol, NEN)]. The cultures were incubated for 12 hr before harvesting.

IL-1 Assay. IL-1 was tested for its ability to induce proliferation of thymocytes (Mizel *et al* 1978). Serial two-fold dilutions of IL-1 were made in 0.1 ml of RPMI containing 10% FCS, 50 mM β-ME and 0.5% penicillin-streptomycin, in flat-bottomed microtiter plates (Linbro). Thymuses from 4- to 6-wk-old C3H/HeJ mice were removed, and a single cell suspension of thymocytes was prepared by mincing the tissue and passing cells through stainless steel wire mesh into RPMI. Cells were washed once and suspended in RPMI containing 5 μg PHA/ml (GIBCO). 1.5 x 10^{6} thymocytes in 0.1 ml of medium were added to each well. Control wells contained medium or IL-1 only. The plates were then incubated for 72 hr, pulsed with 2μCi of methyl-[^{3}H]-thymidine, and harvested after 4 hr.

Measurement of Proliferative Response by ^{3}H-Thymidine Incorporation. Cells were cultured in 96-well microliter plates, and 5-8 hr before the termination of cultures 2 μCi of ^{3}H-thymidine (2 μCi/mMol) were added to each culture. The cells were harvested and the radioactivity was measured in scintillation fluid.

Measurement of ^{3}H-Thymidine, ^{3}H-Uridine, and ^{3}H-Leucine Incorporation for Kinetic Studies. RFC (3 x 10^{3} cells in 0.2 ml of medium) were cultured in 96-well microtiter plates in triplicate as described above. At several time points, the plates were centrifuged and the cells resuspended in 0.2 ml Click's medium without leucine and methionine for the purpose of ^{3}H-leucine incorporation measurements, or in medium without nucleic acids for measurement of ^{3}H-uridine and ^{3}H-thymidine incorporation. Each culture was pulsed with 2 μCi of ^{3}H-thymidine (2 μCi/mMol), ^{3}H-uridine (45.9 μCi/mMol, NEN), or ^{3}H-leucine (56.5 μCi/mMol, NEN). After 4 hr, plates were centrifuged, pelleted cells resuspended in PBS and harvested in 10% trichloroacetic acid (TCA). Radioactivity was measured in scintillation fluid.

Antibodies. The IgG fraction of goat anti-mouse IgM (μ chain-specific, Cooper Biomedical Inc., Malvern, PA) was affinity-purified by chromatography on a Sepharose column (Pharmacia) coupled to mouse IgM (Miles Laboratories Ltd., Rexdale, Ontario, Canada). The affinity-purified antibody was eluted in 1 M acetic acid (pH 2.0), neutralized immediately with Tris pH 8.0, and concentrated to 0.3 to 1 mg/ml before dialyzing against PBS. The specificity of antibody was assessed by radioimmunoassay (RIA) using mouse IgM and mouse IgG (Miles). The affinity-purified goat anti-mouse IgM was specific for IgM and did not bind to IgG as determined by RIA. Similarly, affinity-purified goat anti-mouse IgG was specific for IgG. Goat anti-mouse IgG (Fc-portion-specific) was likewise purified and used as negative control.

Radioiodination of Antibodies. Affinity-purified antibodies were labelled with Na^{125}I by the chloramine T method (Greenwood *et al*, 1963). 100 μg of protein in 100 μl PBS (1 mg/ml), 0.1 to 0.4 mCi ^{125}I (Edmonton Radiopharmaceutical Centre, University of Alberta, Edmonton, Canada), to which 50 μl of 2 mg/ml chloramine T in PBS were added (Eastman), were incubated for 3 min at room temperature. The reaction was stopped with 50 μl of 6 mg/ml sodium metabisulphate in PBS and the sample transferred to dialysis tubing with 1 ml of 1 M KI. The mixture was dialyzed against several changes of PBS.

Radioimmunoassay. A solid phase RIA was developed to detect total IgM secreted into culture by affinity-enriched B cells stimulated with various combinations of factors. Plastic microtiter plates (Fisher) were coated with 125 pg/well of affinity-purified goat anti-mouse IgM (Cappel) and incubated for 2 hr at room temperature. The wells were then washed once with 5% FCS in PBS and twice with PBS containing 0.005% Tween-20 (Sigma) and 0.02% sodium azide. Culture supernatants from 3 x 10^{3} RFC/well, cultured in 96-well microtiter plates, were obtained by spinning plates at 1200 rpm for 8 min. These supernatants were diluted in RIA buffer (PBS containing 5% bovine serum alubumin (BSA), and 0.02% sodium azide) and 100 μl added to each well. After two hours, the wells were washed several times with PBS-Tween-azide and incubated for 1 hr with ^{125}I-labelled goat anti-mouse IgM (10^{5}-10^{6} cpm/well) at room temperature. The plates were then washed 3-4 times with

PBS-Tween and individual wells cut out and counted in a gamma counter. Twelve-point standard curves were run using standard mouse IgM. The lower limit of detection is about 0.02 μg/ml. The specificity of the iodinated antibody was determined by testing its binding to plates coated first with goat anti-mouse IgG followed by different amounts of mouse IgG.

Antigen-specific IgM (B^2-CRBC-specific) secreted in the supernatant of cultures was assayed as follows. Culture supernatants were diluted and 100 μl added to each well containing 50 μl of 0.4% B^2 haplotype CRBC, in 96-well V-bottom microtiter plates (Dynatech Lab, Virginia). All dilutions were made in PBS supplemented with 1% BSA and 0.02% sodium azide, and plates were incubated with doubling dilutions of CH4, a monoclonal IgM antibody to chicken MHC antigen of B^2 haplotype, to obtain twelve-point standard curves. CH4 was kindly provided by Dr. B.M. Longenecker in our department. The cells were washed 3 times with PBS-Tween-azide buffer by spinning at 2000 *g* for 3 min and incubated for 2 hr with ^{125}I goat anti-mouse antibody (10^5-10^6 cpm/well).

The cells were washed 3 times, resuspended in PBS, transferred to RIA tubes (Fisher), and counted in a gamma counter. The specificity of the culture supernatants was determined by testing its binding to CRBC of a different MHC haplotype, i.e., B^{21}.

Nonspecific Esterase Staining. The adequacy of monocyte depletion was determined by staining for nonspecific esterase by the α naphthyl acetate method described by Davis and Ornstein (1959). 1.1 x 10^6 to 2 x 10^6 normal spleen cells or 11 x 10^4 to 2 x 10^4 rosettes (RFC or RFC 4') were resuspended in 100 μl of culture medium and spun on slides by using cytospin at 300 rpm for 3 min. The slides were routinely stained by the Hematology Unit at the Department of Laboratory Medicine, University of Alberta.

Plaque Assay for IgM-Secreting Cells. The number of PFC was determined by the direct hemolytic plaque assay of Cunningham and Szenberg (1968). Cells suspended in 0.2 ml of medium were cultured in triplicate in 96-well microtiter plates with various combinations of factors. At various time points, microtiter plates containing the cell cultures were centrifuged, washed once with Mishell-Dutton Balanced Salt Solution, and resuspended in 100 μl MD-BSS. Fifty μl of 5% CRBC and 50 μl of guinea pig complement (diluted 1:6 in MD-BSS) from GIBCO were added to each well and mixed thoroughly using a tygan tubing-tipped pasteur pipette. Fifty μl from each well were loaded into a 3-chambered Cunningham slide and sealed with paraffin vaseline sealant (1:1 by weight). The slides were incubated for 60 min at 37 °C and plaques counted at low magnification. Results are expressed as PFC per culture and represent the arithmetic mean of triplicate cultures ± standard error.

Fluorescent Staining. 1 x 10^6 to 2 x 10^6 normal spleen cells or 0.5 x 10^6 rosettes (RFC or RFC 4') were washed with wash buffer (PBS containing 2% FCS and 0.1% sodium azide). The pellet was then resuspended in 50 μl of appropriately diluted antibody (Miles, fluorescein-conjugated F(ab')$_2$ fragment of goat anti-mouse IgM or IgG, rhodamine-conjugated F(ab')$_2$ fragment of goat anti-mouse Thy-1.2) in PBS containing 0.1% sodium azide. After 1 hr incubation on ice, the cells were washed three times with wash buffer and resuspended in 25 μl of wash buffer and 25 μl of 2% formalin (v/v in PBS).

Plasmid Preparation. HB101μ12 was grown on L broth. Before plasmid preparation, the drug sensitivity of bacteria was confirmed. HB101μ12 is ampicillin sensitive and tetracycline resistant. Large scale plasmid preparation was done by the method of Birnboim (1983). Bacteria were grown overnight in L broth at 37 °C in the presence of tetracycline at a final concentration of 10 μg/ml. A 100 μl innoculum from the saturated culture was added to 500 ml X broth containing tetracycline (GIBCO) and the bacteria grown to an OD$_{650}$ of 0.9. Five hundred ml of X broth containing 600 mg spectinomycin (GIBCO) were added and the cultures maintained overnight. The bacteria were killed by addition of 10 ml chloroform and stirred for 10 min at 37 °C prior to centrifugation at 5000 *g* for 10 min. The pellet was resuspended in 15 ml TE buffer pH 7.5 (20 mM Tris-HCl, 1 mM ethylenediaminetetraacetic acid [EDTA]) and centrifuged at 5000 rpm for 10 min. The pellet was resuspended in 15 ml of 50 mM Tris-HCl (pH 7.5), treated with 2.5 mg/ml lysozyme (GIBCO), mixed gently, and left on ice. After 10 min, 930 μl of 2% Triton-X 100 (Sigma) were added and the mixture left standing on ice for 60 min. The cell debri was removed by centrifugation at 30 K for 45 min at 4 °C. The supernatant was collected and adjusted to a pH of 12.45-12.5 with 5 M NaOH. After 10 min, the pH was brought back to 8.5 with 3 M Tris-HCl (pH 7.5). The mixture was allowed to stand at room temperature for 3 min and one-ninth volume of 5 M

NaCl added. The pellet was resuspended in 3 ml of 0.04X saline sodium citrate (SSC) and sodium dodecyl sulphate (SDS) added to a final concentration of 0.04%. Thereafter, the samples were treated with proteinase K (1 mg/ml final concentration, BRL) for 30 min at 37 °C. The mixture was extracted three times with phenol and twice with chloroform after which NaCl was added to a 0.3 M final concentration. The sample was precipitated with 100% ethanol at -20 °C. The pellet obtained was washed gently with 80% ethanol, resuspended in TE buffer (10 mM Tris pH 7.5, 10 mM NaCl, 0.1 mM EDTA), and stored frozen at -20 °C till use.

Preparation of the μ Insert. The double-stranded cDNA segment was inserted into the *Pst* site of the recombinant plasmid p104Eμ12. To obtain the Cμ-encoding sequence from p104Eμ12, the plasmid preparation was digested with *Pst* 1 (Pharmacia) under digestion conditions specified by Maniatis *et al* (1982). Digestion products were analyzed on 0.7% agarose gel (GIBCO). The Cμ insert (residue 300 to 576) contained a *Pst* site in the Cμ4-encoding region. The three bands obtained upon digestion represented the plasmid DNA (large fragment, slow moving band) and μ insert (two small fragments or fast moving bands). The two smaller fragments were eluted from agarose gel as described by Maniatis *et al* (1982), ethanol precipitated, and utilized as the μ probe.

Preparation of Cytoplasmic RNA. Cytoplasmic RNA was prepared from RFC at 4.5 days of culture by modification of the method described by Maniatis *et al* (1982). The RFC were layered over Ficoll to remove CRBC. The cells were washed with PBS and microfuged for 2.5 min at 4 °C. The pellet (1-10 x 10^6 cells) was resuspended in 45 μl lysis buffer consisting of 0.14 M NaCl, 1.5 mM $MgCl_2$, 10 mM Tris-Cl (pH 8.6), and 10 mM vanadyl-ribonucleoside complex (NEN) as an inhibitor of RNase. The cells were lysed with 5 μl of 5% Nonidet P-40 (NP-40) twice with 5 min intervals on ice and centrifuged at 4 °C. After 2.5 min, the pellet was discarded and the supernatant transferred to a fresh tube with an equal volume of 2X proteinase K buffer (0.2 M Tris-Cl pH 7.5, 25 mM EDTA, 0.3 M NaCl, 2% w/o SDS) and proteinase K (Fungal, BRL) to a final concentration of 0.2 mg/ml. The mixture was incubated at 37 °C for 30 min followed by a phenol:chloroform extraction. The aqueous phase recovered by centrifugation for 15 min was precipitated with 2.5 vol of

98% ethanol and left at -20 °C overnight. The pellet obtained by centrifugation for 15 min at 4 °C was washed gently with 80% ethanol containing 0.1 M sodium acetate (pH 5.2), microfuged for 15 min, and resuspended in the least volume of TE buffer.

Electrophoresis of RNA Samples. A modification of the method described by Maniatis *et al* (1982) was used for electrophoresis of RNA samples. The RNA gel apparatus, glassware, and tubing for the peristaltic pump were treated with diethyl pyrocarbonate (DPC, Sigma) overnight (2 ml of DPC dissolved in 50 ml of 95% ethanol and made up to 1 litre in DDW) and rinsed thoroughly with autoclaved DDW before use. The solutions required were:

1. The 5X gel buffer autoclaved and stored protected from light.

   0.2 M 3-[Morpholino]propane sulfonic acid (MOPS) (pH 7.0)
   50 mM sodium acetate
   5 mM EDTA (pH 8.0)

2. Formamide (Fisher) was deionized using Bio Rad AG501-X8 until the pH was neutral. It was then aliquoted and stored at -20 °C.
3. Formaldehyde (Terochem Lab Ltd., Canada) was a 37% solution in water with pH greater than 4.0.
4. Loading buffer:

   50% glycerol
   1 mM EDTA
   0.4% Bromo phenol blue
   0.4% Xylene cyanol FF dye (Biorad)

A 1% agarose gel containing 20% 5X gel buffer and 21.7% formaldehyde was made in a horizontal gel apparatus (Tyler Research Corporation, NC). RNA samples for loading were prepared by mixing:

4.5 μl RNA sample (up to 20 μg)
2.0 μl 5X gel running buffer
3.5 μl formaldehyde
10.0 μl formamide

2.0 μl loading buffer

and incubation for 10 min at 65 °C. Samples and RNA standards were loaded on the gel immediately afterwards. The gel was run in 1X gel buffer with buffer stirring continuously in the two tanks that were connected by a peristaltic pump. The gel was run at 50 volts for 15 hr.

Transfer of RNA From Agarose to Nitrocellulose. After electrophoresis, the gel was soaked for 5 min in several changes of water at room temperature. At this time, the gel is not rigid and liable to break. The gel was photographed after staining with 1 μg/ml ethidium bromide (in DDW) for 1 hr at room temperature. Later, the gel was neutralized by soaking for 45 min in 0.1 M Tris-Cl (pH 7.5) at room temperature and soaked for 1 hr in 20X SSC before transfer to nitrocellulose paper (Millipore Corp.). The transfer of RNA from agarose gels to nitrocellulose was performed as described by Maniatis *et al* (1982). The nitrocellulose paper was soaked in 3X SSC. The concentration of the transfer buffer was 20X SSC. After 24 hr, the nitrocellulose paper was washed in 3X SSC for 90 min in a 65 °C waterbath. This was followed by another wash in 3X SSC containing 10X Denhardt's solution for 3 hr or overnight at 65 °C.

Labelling by the Oligo-Labelling Method. The oligo-labelling reaction (Feinberg and Vogelstein, 1983) was used to radiolabel DNA probes for hybridization in northern blots. A 25 μl reaction mixture was prepared as follows in the order mentioned:

11.4 μl LS
1.0 μl BSA (10 mg/ml, BRL-Laboratories)
2.5 μl (0.1μg) plasmid DNA (boiled to 100 °C for 3 min and cooled immediately)
4.1 μl DDW
5.0 μl α^{32}P-CTP (Amersham Corp, Ont.)
1.0 μl klenow fragment (BRL, 4.5 U/ml)

The solutions required for oligo-labelling are:

LS = 25 parts of 1 M Hepes + 25 parts of DTM + 7 parts of OL (by volume)
DTM = 100 μM of deoxyadenosine triphosphate, deoxycytosine triphosphate (dCTP), deoxyguanosine triphosphate, deoxythymidine triphosphate in Tris-buffer,

pH 8.0 (250 mM Tris-HCl, 25 mM $MgCl_2$ and 50 mM β-ME)

OL = 1 mM Tris (pH 7.5) and 1 mM EDTA (pH 7.5) and 90 OD units/ml

OD = oligodeoxyribonucleotide (Pharmacia).

The components were gently mixed, centrifuged briefly, and incubated at room temperature. The incorporation of radioactive precursors was monitored by acid-precipitation. One μl aliquot of the reaction mixture was spotted on small pieces of DE-81 (Fisher) filter discs and counted using a β counter or Geiger counter. Subsequently, the acid precipitable counts were estimated by dropping the discs in 12% cold TCA. The percentage of incorporated radioactive precursors was calculated from TCA precipitable counts as compared to total counts. The percentage of ^{32}P-dCTP incorporated into DNA usually achieved 90% by 3 hr. At this time, 1 μl of heat-denatured tRNA was added to the reaction mixture and the mixture extracted with an equal volume of phenol by microfuging for 3 min. The aqueous phase was immediately transferred to another tube. The radiolabelled DNA fragments were recovered by loading them on a Sephadex G-50 (in Tris-EDTA pH 7.5) column followed by elution with TE (pH 7.5). Seven to eight fractions of 300-400 μl each were collected from the column. Earlier fractions contained the labelled DNA while later fractions consisted of free radioactive precursors, thus giving a bimodal peak on plotting cpm *vs* fraction number. The high radioactive fractions of the 1st peak were pooled, and the specific activity determined by counting 1 μl aliquots in 1 ml aquasol-2 scintillation fluid using a β counter. Over 70% of the precursor triphosphate was routinely incorporated into complementary DNA and specific activities of 10^8 cpm/μg of DNA were obtained using this procedure.

Cytoplasmic Dot Hybridization. RFC were cultured in petri dishes at a cell density of 1.5 x 10^6 cells/ml and run over Ficoll to remove CRBC. The cells were washed twice with PBS and used for cytoplasmic preparations as described by White and Bancroft (1982). Briefly, 10^6-10^7 cells were pelleted by centrifugation for 15 seconds in a 1.5 ml Eppendorf tube in an Eppendorf model 3200 centrifuge. The cells were resuspended in 45 μl cold 10 mM Tris (pH 7.0), 1.0 mM EDTA, lysed by adding 5 μl of 5% NP-40 (Sigma) twice, and mixed on ice for 5 min. After 5 minutes, nuclei were pelleted (2.5 min in Eppendorf centrifuge) and 50 μl supernatant transferred to Eppendorf tubes containing 30 μl of 20X SSC and 20 μl of 37% (w/o) formaldehyde (Terochem Lab Ltd., Edmonton, Canada). The mixture was incubated for 15 min at 60 °C and either dotted or frozen at -70 °C for subsequent analysis. At a later time, the mixture was incubated for 5' at 60 °C, mixed, and used. A minifold apparatus (Schleicher and Schuell, Keene, NH) was employed to apply denatured cytoplasm to BA85 nitrocellulose (from S and S). The BA85 nitrocellulose was prewetted in DDW followed by 15X SSC. Serial dilutions of cytoplasm were made in 96-well microtiter plates containing 15X SSC in a final volume of 150 μl. The first dilution of sample contained the equivalent from 3 x 10^5 cells in a maximum volume of 20 μl. The high salt concentration used enables RNA to bind to nitrocellulose. Only 100 μl aliquots of each sample were applied to nitrocellulose. Following application, the nitrocellulose was baked in a vacuum oven at 80 °C for 90 min.

Hybridization and Autoradiography. The RNA from gels was transferred to nitrocellulose paper as described earlier (Maniatis *et al* 1982). Both RNA blots and RNA gel filters were prepared for hybridization by washing in 3X SSC at 65 °C for 90 min and then with 3X SSC containing 10X Denhardt's solution, at 65 °C for 3 hr or overnight.

The hybridization mixture was prepared as follows in a final volume of 600 μl:

120 μl 50X Denhardt's solution
120 μl 5 M NaCl
30 μl M Tris pH 8.0
12 μl 0.5 M EDTA
1.2 μl 5 mg/ml *E. coli* DNA (Pharmacia)
6 μl 5 μg/ml yeast tRNA (Pharmacia)
6 μl 10% SDS
300 μl Probe [5 x 10^7 cpm or 0.04 μg]
4.8 μl DDW

The *E. coli* DNA, yeast tRNA, and the labelled probe were heated at 100 °C for 3 min and cooled immediately. The nitrocellulose filter and the above solution mix were sealed in a plastic bag and left overnight at 65 °C. The filter was later washed with 4-5 changes of 2X SSC, the first and second washes for 2-3 min each, the third wash for 15 min, and the final two washes for 2-3 min each. After washing, the filter was soaked in 0.1X SSC for 1 hr at 65 °C and washed again with 2X SSC twice for 2-3 min each time. The filter was blotted dry, placed on Whatman #3 paper, wrapped with saran wrap, and exposed to X-ray film (Kodak XAR-2) for ten days at -70 °C.

III. Results

This thesis examines some aspects of the complex phenomenology of B cell activation. Three main themes have emerged from these studies.

Firstly, a model system of B cell activation is described. A polyclonal activator, LPS, stimulates antigen affinity-enriched B cells to proliferate. Differentiation of B cells into antibody-secreting cells requires the addition of LPS and T cell lymphokines such as supernatant from the PMA-induced EL-4 line. Secondly, the correlations between early biochemical changes and B cell activation was investigated. Finally, the role of r IL-2 and r IL-1 in this model of B cell activation is defined.

A. Regulation of B Cell Activation by LPS and EL-4 Sup

Earlier studies have demonstrated that mice immunized with CRBC of the B^2 MHC haplotype generate a much higher PFC response to B^2 alloantigen than to CRBC bearing any other MHC product (Longenecker *et al*, 1979). Initiation of an *in vitro* CRBC-specific IgM response by unprimed mouse spleen cells required antigen, antigen-specific T_h cells, and adherent cells. T_h cells and adherent cells could be replaced by products extracted from or secreted by these two cell classes, respectively (Shiozawa *et al*, 1980). Experiments using LPS as a nonspecific activator rather than helper factor derived from antigen-primed T cells, showed that LPS-stimulated B cells undergo marked proliferation without any increase in B^2-CRBC-specific PFC. Generation of CRBC-specific PFC by LPS-stimulated B cells required the presence of A cells, culture supernatants of A cells, or partially purified culture supernatants from PMA-stimulated EL-4 (Jaworski *et al*, 1982). In the presence of B^2-CRBC-specific helper factor, EL-4-derived supernatant could not promote a PFC response unless A cells were also present (Shiozawa *et al*, 1984). The experimental model for B cell activation used in this investigation is derived from the observations made in the above-mentioned studies.

Enrichment of B Cells for Antigen-Specific Response

B cells recognizing chicken B^2 MHC alloantigen were enriched by virtue of their property to form rosettes with CRBC (Figure 1). The ability of spleen cells to rosette was completely inhibited by pretreating them with anti-mouse Ig antibody but not by treating them with anti-Thy-1 antibody (Shiozawa *et al*, 1980). By using the antigen-rosette technique, the number of Ig-bearing RFC could be enriched 500- to 1000-fold. The routine preparations of RFC were obtained by centrifugation through Ficoll for 25 min to enrich RFC and to remove a significant proportion of large, low-density cells. These RFC preparations contained rosettes as well as varying numbers of non rosetted cells contaminating rosettes. These contaminating cells are usually retained in the interphase during the Ficoll spin. To circumvent contamination of nonrosetted cells in the RFC preparation, the time of centrifugation in Ficoll was reduced. An RFC population containing less than 10:1 contamination of nonrosetted with rosetted cells was obtained upon 4 min centrifugation and will be referred to as RFC 4'. The properties of RFC 4' will be discussed in a subsequent section.

RFC and RFC 4' were cultured without removing the antigen in the form of CRBC surrounding the RFC.

Proliferation and Differentiation of RFC Depends Upon LPS and EL-4 Sup, Respectively

RFC were cultured in varying concentrations of LPS in Click's medium containing 5% FCS and other supplements (see *Materials and Methods*) in the absence of EL-4 sup, and the level of proliferation measured after 4 days of culture. The proliferative response was maximal after 4 days of culture (data not shown, see Figure 13). Stimulation of RFC with 10 μg LPS/ml of culture resulted in an optimal proliferative response (Figure 2). Culturing varying numbers of RFC demonstrated that 3 x 10^3 RFC/culture were sufficient to exhibit profound proliferation in 10 μg/ml of LPS (Figure 3). LPS alone, in the absence of other stimuli, did not induce B^2-CRBC-specific PFC (Figure 4). As shown below, the induction of antigen-specific PFC requires the presence of both LPS and EL-4 sup, and neither LPS or EL-4 sup alone can induce PFC.

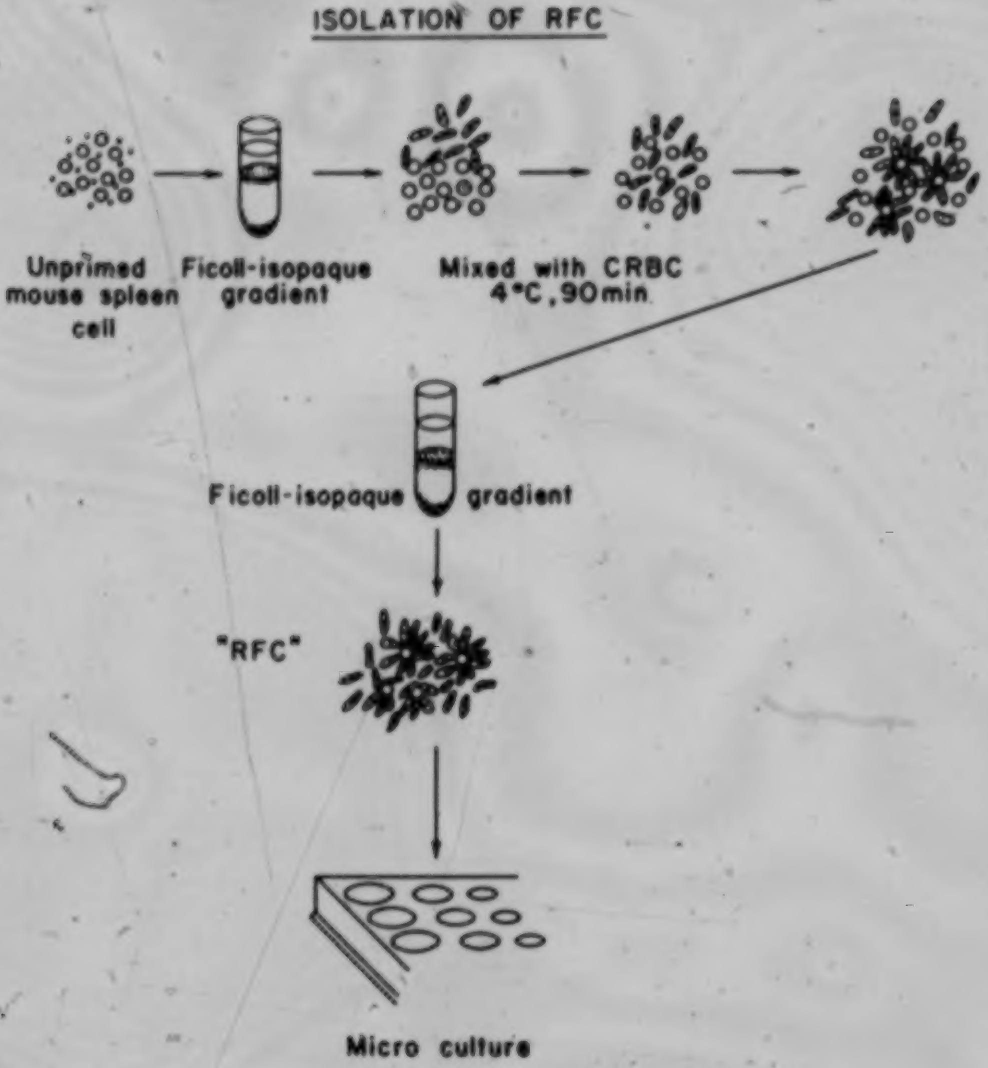

ISOLATION OF RFC
Unprimed mouse spleen cell
Ficoll-isopaque gradient
Mixed with CRBC 4°C, 90min
Ficoll-isopaque gradient
"RFC"
Micro culture

Figure 1. Illustration of rosette preparation.

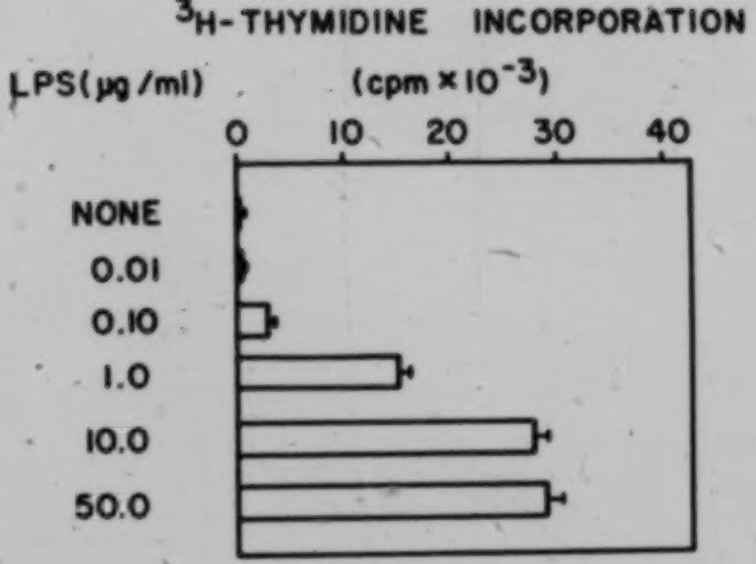


Figure 2. Effect of LPS on proliferation. RFC (3 x 10^3/well) were cultured with various concentrations of LPS for 4 days, pulsed with 2 µCi ^{3}H-thymidine for 4-6 hr, and harvested. Data represent means of triplicate experiments and results are expressed in cpm/culture ± S.D.

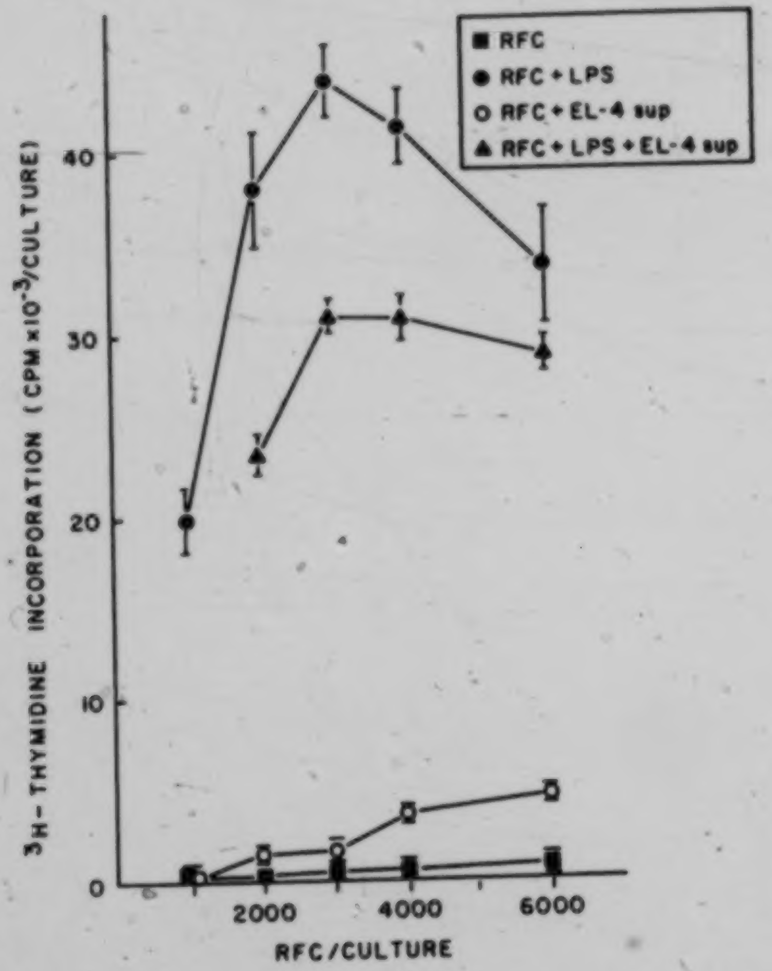


Figure 3. Effect of different concentrations of RFC on the activation of proliferation induced by LPS and/or EL-4 sup. RFC were cultured at various cell concentrations with 10 μg/ml of LPS and/or 1:20 dilution of EL-4 sup. On the 4th day, the cultures were pulsed with ³H-thymidine for 4 hr and harvested. Data represent means of triplicate experiments and results are expressed as cpm/culture ± S.D.

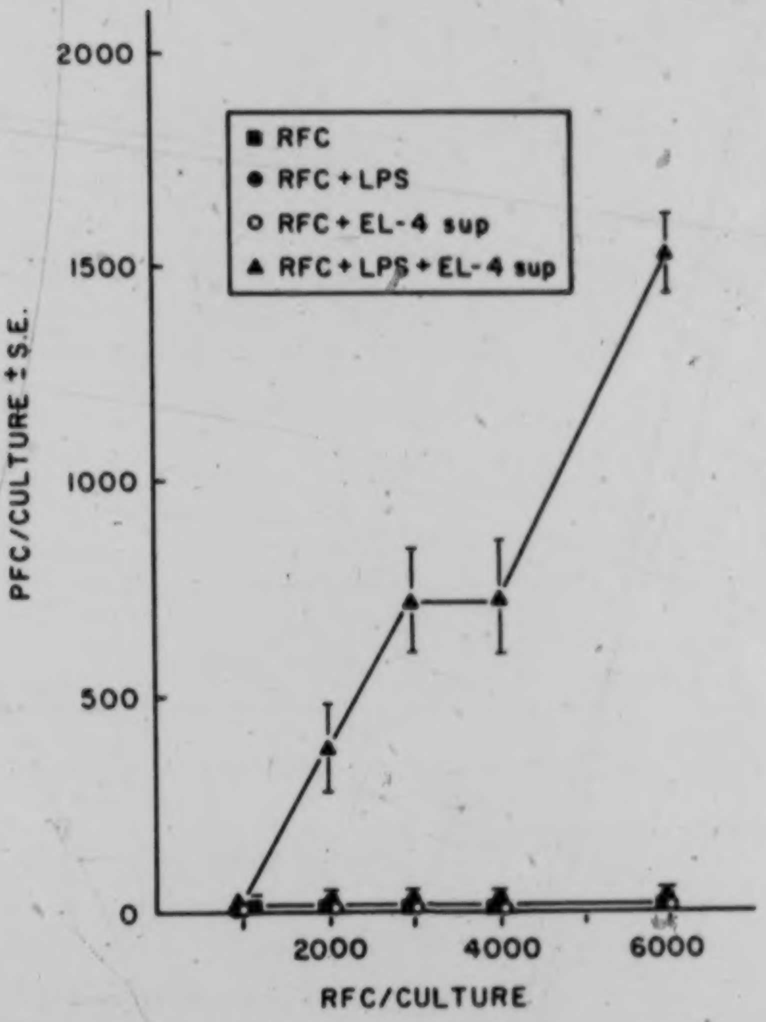


Figure 4. Effect of different concentrations of RFC on the generation of a PFC response with LPS and/or EL-4 sup. RFC were cultured at various cell numbers with 10 μg/ml of LPS and/or 1:20 dilution of EL-4 sup. Cultures were assayed for anti-*B'* IgM plaques on day 4.5. Data represent means of triplicate experiments and results are expressed as PFC/culture ± S.E.

The cell-free culture supernatant of the EL-4 line obtained after a 24 hr culture with PMA, was ammonium sulfate precipitated and dialyzed before use (see *Materials and Methods*). Since LPS does not stimulate RFC to mature into antibody-secreting cells, we tested the ability of EL-4 sup to induce PFC. EL-4 sup alone does not induce proliferation (Figure 5) or differentiation (Table 1) of RFC. Only at very high concentrations of EL-4 sup is there marginal proliferation of RFC. EL-4 sup at a dilution that induces minimal proliferation of RFC (1:20 dilution from Figure 5), was shown to generate maximum number of PFC (Table 1). An optimal PFC response was obtained with 3 x 10^5 RFC/well, in the presence of 10 μg/ml of LPS and a 1:20 dilution of EL-4 sup (Figure 4). Increase in PFC response obtained with 6 x 10^5 RFC/well may be due to additional effect of contaminating cells at high cell densities.

The Antibody Response Generated by LPS and EL-4 Sup is Antigen Specific

A PFC response specific for CRBC requires the presence of antigen, LPS, and EL-4 sup in culture. Spleen cells, which are not enriched for B cells, do not generate CRBC-specific PFC on activation by LPS and EL-4 sup (Table 2). To produce a CRBC-specific PFC response, antigen needs to be added to the culture. The PFC produced are not specific for the MHC haplotype of CRBC used for stimulation (Table 2). Enriching for B cells by anti-Thy-1.2 and complement treatment, and subsequent culture with B^2-CRBC, LPS, and EL-4 sup, again generated PFC responses to other alloantigens of CRBC (Table 3). The PFC response obtained by stimulating RFC with LPS and EL-4 sup was significantly specific for the MHC haplotype of the stimulating CRBC (Table 4).

Effect of Macrophage and T Cell Depletion on B Cell Activation

A number of soluble factors produced by T lymphocytes and macrophages influences the growth and maturation of B lymphocytes (discussed in *Chapter I*). Contamination of B cell preparations with small numbers of T cells or macrophages may lead to misinterpretation of the precise signals required for B cell activation. To more precisely delineate the requirements for B cell activation, the effect of macrophage and T cell depletion prior to

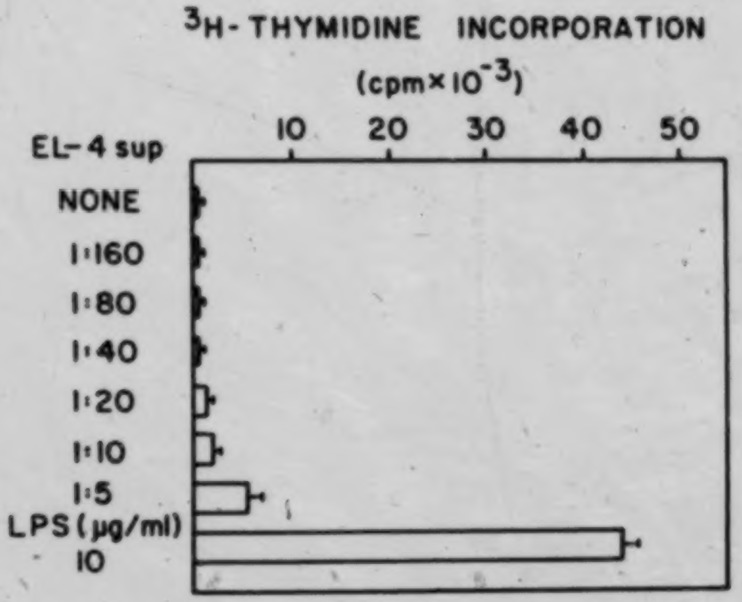


Figure 5. Effect of EL-4 sup on proliferation. RFC 3 x 10^5/well were cultured with various dilutions of EL-4 sup for 4 days, pulsed with 2 μCi ^{3}H-thymidine for 4-6 hr and harvested. Data represent mean of triplicate experiments and the results are expressed as cpm/culture ± S.D.

Table 1

Effect of EL-4 Sup on the Induction of PFC

Addition[1]	EL-4 Sup Concentration[2]					
	0	1:80	1:40	1:20	1:10	1:5
None	<1	<1	<1	<1	<1	<1
LPS	36 ± 6	164 ± 68	524 ± 32	844 ± 8	124 ± 28	108 ± 42

[1] RFC (3 x 10^5 cells/well) were cultured in the absence or presence of 10 μg/ml of LPS.

[2] Various concentrations of EL-4 sup were added at the onset of culture. Cells were assayed for anti-B^2 IgM PFC at the end of 4.5 days. Data represent means of triplicate cultures and the results are expressed as PFC/culture ± S.E.

Table 2

Specificity of PFC Response with Spleen Cells

Stimulant	IgM Plaque-Forming Cells[2]					
	Spleen Cells[1]		Spleen Cells + B^2-CRBC		Spleen Cells + B^{14}-CRBC	
	Anti-B^2	Anti-B^{14}	Anti-B^2	Anti-B^{14}	Anti-B^2	Anti-B^{14}
None	<1	<1	<1	<1	<1	<1
LPS	<1	<1	<1	<1	<1	<1
EL-4 Sup	<1	<1	<1	<1	<1	<1
LPS + EL-4 Sup	<1	<1	1,076 ± 141	1,320 ± 119	1,710 ± 326	1,332 ± 20

[1] Spleen cells were cultured for 4.5 days at 10^6 cells/well in the presence of LPS, EL-4 sup, and B^2-CRBC or B^{14}-CRBC.

[2] The cultures were assayed for anti-B^2- and anti-B^{14} IgM PFC. The results represent means of triplicate experiments and the results are expressed as PFC/culture ± S.E.

Table 3

Specificity of PFC Response with T-Depleted Spleen Cells

Stimulant	IgM Plaque-Forming Cells[2] from:					
	T-Depleted Spleen Cells[1]		T-Depleted Spleen Cells + B^2-CRBC		T-Depleted Spleen Cells + B^{14}-CRBC	
	Anti-B^2	Anti-B^{14}	Anti-B^2	Anti-B^{14}	Anti-B^2	Anti-B^{14}
None	<1	<1	<1	<1	<1	<1
LPS	<1	4 ± 2	<1	<1	<1	8 ± 6
EL-4 Sup	<1	<1	<1	10 ± 5	<1	44 ± 6
LPS + EL-4 Sup	<1	17 ± 7	1,070 ± 145	928 ± 164	504 ± 20	572 ± 41

[1] Spleen cells depleted of T cells as discussed in *Materials and Methods*, were cultured for 4.5 days at 10^6 cells/well in the presence of LPS, EL-4 sup, and B^2-CRBC or B^{14}-CRBC.

[2] The cultures were assayed for anti-B^2 and anti-B^{14} IgM PFC. The results represent means of triplicate experiments and results are expressed as PFC/culture ± S.E.

Table 4

Specificity of PFC Response with RFC

Stimulant	IgM Plaque-Forming Cells[2] from:			
	RFC[1] B^2		RFC B^{14}	
	Anti-B^2	Anti-B^{14}	Anti-B^2	Anti-B^{14}
None	<1	<1	<1	<1
LPS	<1	<1	<1	<1
EL-4 Sup	<1	<1	<1	<1
LPS + EL-4 Sup	1,888 ± 314	534 ± 81	1,083 ± 89	2,760 ± 293

[1] RFC were cultured at 3000 cells/well in the presence of LPS and EL-4 sup for 4.5 days.

[2] The cultures were assayed for anti-B^2 and anti-B^{14} IgM PFC. The results represent means of triplicate experiments and are expressed as PFC/culture ± S.E.

rosetting with CRBC was studied. Moreover, the EL-4 sup used in these studies contains IL-2 (discussed in Farrar *et al*, 1982), and there was a possibility that the effect of EL-4 sup on inducing PFC may, in fact, have been due to stimulation of contaminating T cells in RFC preparations. Depletion of adherent cells and T cells prior to rosetting decreased the number of PFC obtained but the stimulation index upon stimulation with LPS and EL-4 sup, remained the same (Table 5). Control experiments showed that T cell depletion by treatment with anti-Thy-1.2 monoclonal antibody and complement abolished the proliferative response to Con A. These results further confirm the observation that stimulation of RFC by LPS and EL-4 sup involves B cells and that the culturing of RFC at low cell numbers overcomes the problems associated with contaminating non-B cells.

Both LPS and EL-4 Sup are Required During the Early Phase of B Cell Activation

The kinetics of addition of LPS and EL-4 sup to cultures and its effect on proliferation are shown in Table 6. LPS was effective in inducing proliferation of RFC when added at the onset of culture. However, after 2 days, addition of LPS induced drastically less proliferation and by 3 days, the RFC could no longer be stimulated with LPS. This effect may reflect loss in viability of RFC with time. The viability of RFC in culture drops sharply to less than 70% by 40 hr in the absence of a stimulant (Figure 6). Since EL-4 sup alone did not induce significant proliferation, it had little effect on proliferation when added at different time intervals (Table 6).

Figure 7 shows the time kinetics of addition of LPS and EL-4 sup to cultures and its effect on induction of PFC. Delayed addition of either EL-4 sup to LPS-stimulated RFC or LPS to EL-4 sup-stimulated RFC resulted in a decrease in the PFC response. Addition of LPS to EL-4 sup-stimulated RFC cultures at 40 hr after initiation of cultures failed to induce a PFC response. However, EL-4 sup could be added to LPS-stimulated RFC cultures at 40 hr without a drastic reduction in PFC response. These results indicate that both LPS and EL-4 sup are required during the early phase of B cell activation and LPS is required before EL-4 sup during this response.

Table 5

Effect of Macrophage and T Cell Depletion on Differentiation of RFC

Cell Culture[1]	PFC/culture ± S.E. in the Presence of:[2]			
	None	LPS	EL-4 Sup	LPS + EL-4 Sup
RFC	<1	70 ± 9	<1	1,052 ± 346
Anti-Thy-1.2- and Adherent Cell-Depleted RFC	<1	34 ± 18	<1	572 ± 117

[1] Spleen cells depleted of adherent cells (see *Materials and Methods*) were treated with anti-Thy-1.2 monoclonal antibody and rabbit complement before rosetting. The treatment abolished the proliferative response to Con A.

[2] RFC were cultured at 3 x 10^3 cells/well and assayed on day 4.5 for anti-B^1 IgM PFC. Data represent means of triplicate experiments.

Table 6

Kinetics of Induction of Proliferation by Addition of LPS and EL-4 Sup

Stimulant at Beginning of Culture[1]	Time of Addition (days)	Uptake of ^{3}H-Thymidine by RFC on Addition of:[2]	
		LPS	EL-4 Sup
None	0	14,204 ± 977	899 ± 117
	1	12,293 ± 2,538	811 ± 223
	2	10,825 ± 629	1,460 ± 381
	3	2,682 ± 483	944 ± 78
LPS	0	-	17,536 ± 5,305
	1	-	21,082 ± 3,205
	2	-	22,272 ± 1,799
	3	-	22,764 ± 2,933
EL-4 Sup	0	27,536 ± 5,305	-
	1	16,969 ± 3,818	-
	2	10,391 ± 935	-
	3	4,017 ± 696	-

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[1] RFC were cultured at 3 x 10^5 cells/well in the presence of LPS or EL-4 sup.

[2] At different time intervals, LPS or EL-4 sup was added to RFC cultures. Four-day cultures were pulsed for 4 hr with thymidine and harvested. Results are expressed in cpm/culture ± S.D. RFC cultured without any LPS or LPS and EL-4 sup for 4 days showed a background incorporation of 371 ± 155 cpm.

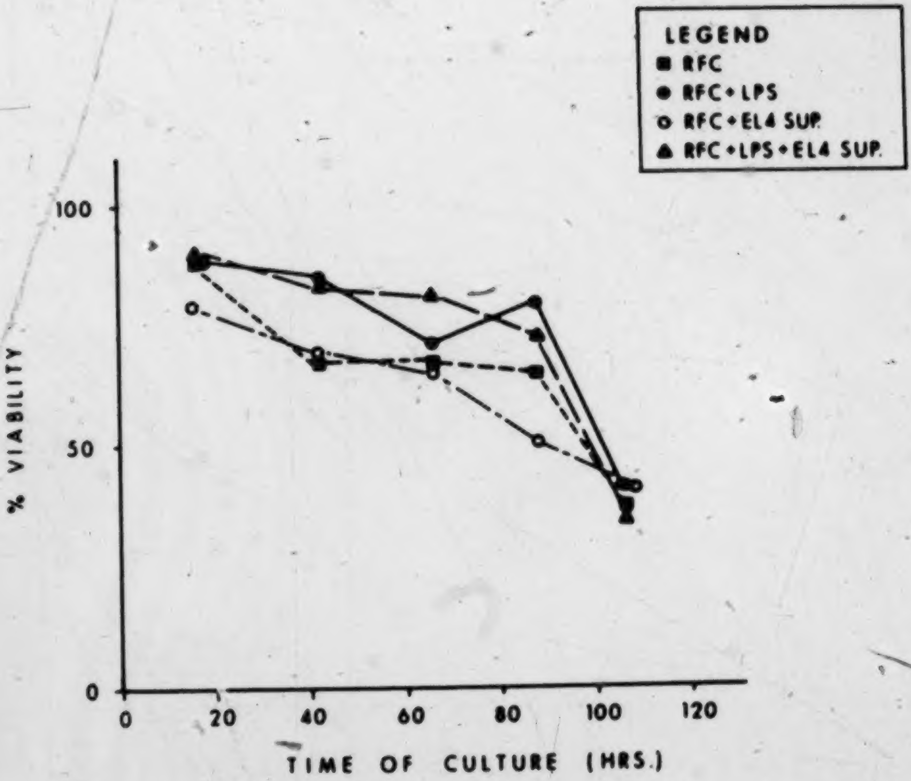

Figure 6. Effect of LPS and/or EL-4 sup on the viability of RFC. RFC were cultured at 3×10^5/well in the absence or presence of stimulants. At several time points, aliquots of cells were removed and tested for viability by the eosin exclusion test.

$$\left(\text{Percentage viability} = \frac{\text{viable cells}}{\text{total cells}} \times 100\right)$$

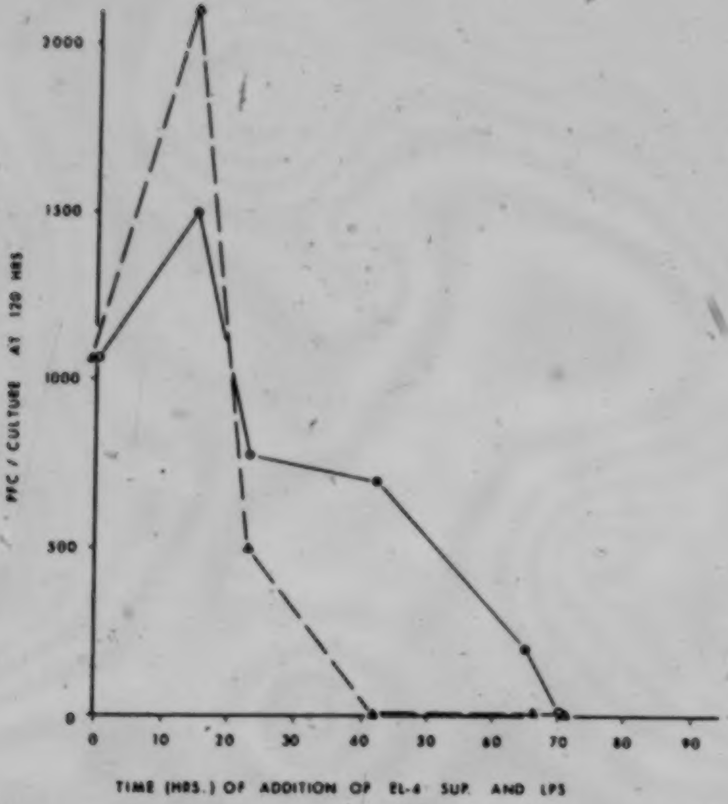

Figure 7. Kinetics of induction of PFC by addition of LPS and EL-4 sup. RFC were cultured at 3 x 10^5/well in the presence of LPS or EL-4 sup. At several time points, EL-4 sup (open circles) or LPS (open triangles) was added to RFC cultures. The cultures were assayed for anti-B^1 IgM PFC at day 4.5. Control cultures of RFC with either LPS or EL-4 sup gave background PFC of 8 ± 6 and <1, respectively. Data represent means of triplicate experiments expressed as PFC/culture ± S.E.

IL-2- and BSF-1-Like Activity of EL-4 Sup

EL-4 sup contains a variety of biological activities including TRF, BSF-1, and IL-2 (Farrar *et al*, 1982; Howard *et al*, 1982). First, we determined if the preparation of EL-4 sup used in these experiments contained any IL-2-like activity. IL-2 was assayed in the EL-4 sup by its ability to induce proliferation of HT-2 cells. IL-2 units were calculated by the method of Hedrick *et al* (1982). This batch of EL-4 sup was used at 10 μl/well of a 1:20 dilution in the presence of LPS for the differentiation of proliferating RFC into PFC. This amounts to 1.2 U of IL-2/well. Therefore, the dilution at which EL-4 sup is used in these experiments contains very little IL-2-like activity.

Next, the BSF-1 activity in EL-4 sup was quantitated. Figure 18 shows a co-stimulator assay for BSF-1 activity. B cells prepared by treating spleen cells with monoclonal antibody to Thy-1.2 and complement were cultured with doubling dilutions of affinity-purified goat anti-IgM F(ab')$_2$. When resting B cells were stimulated with low doses of anti-Ig, the addition of EL-4 sup supported proliferation to a significantly greater degree than did anti-Ig alone, indicating that EL-4 sup contains some BSF-1-like activity. The peak of BSF-1-induced proliferative activity in this assay was seen at 3 days. A purified preparation of BSF-1 was not available for comparison.

Discussion

The mechanisms responsible for proliferation and differentiation of murine B cells were studied using antigen affinity-enriched B cells. To date, a variety of procedures have been developed to select for antigen-binding B cells or to obtain highly enriched populations of B cells. Many methods used for selection of antigen-binding cells, for instance, hapten-gelatin plates (Neale and Cambier, 1981), affinity columns (Truffa-Bachi and Wofsy, 1970; Scott, 1976), flourescence-activated cell sorting (Julius *et al*, 1972), and density gradients (Tanenbaum and Sulitzeanu, 1975), frequently result in inactivation of cells during the enrichment process. Antigen affinity enrichment of B cells by rosetting (described in *Material and Methods*), on the other hand, does not result in loss of functional activity of B cells. Rosetting spleen cells with CRBC selects for a highly enriched population of

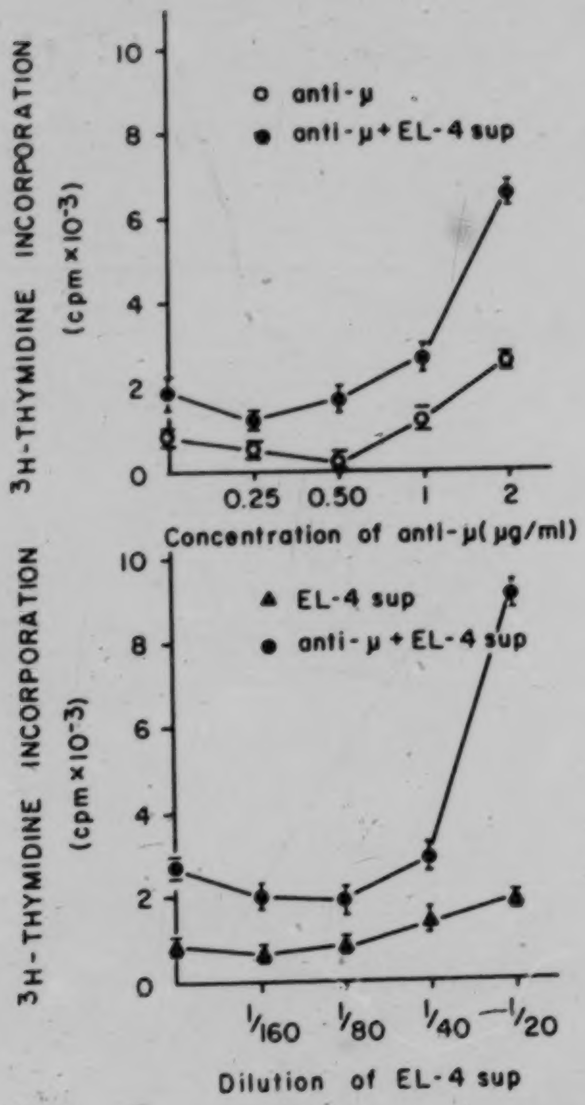

Figure 8. EL-4 sup exhibits BSF-1-like activity. B cells prepared by anti-Thy-1.2 and complement treatment of spleen cells were cultured at 5 x 10^5/well in the presence of (a) varying dilution of $F(ab')_2$ fragment of anti-IgM and a 1:20 dilution of EL-4 sup or (b) varying dilutions of EL-4 sup and 2 μg/ml anti-IgM. At 3 days, the cultures were pulsed with ^{3}H-thymidine for 4 hr and harvested. Results represent means of triplicate experiment expressed as cpm/culture ± S.D.

antigen-binding B cells as judged by flourescent microscopy (99 percent of rosettes contain IgM$^+$ B cells), the ability of anti-mouse Ig antibodies to inhibit rosette formation (Shiozawa, 1980), and the demonstration of proliferation and differentiation of these cells by a combination of stimuli *in vitro*. By using CRBC as antigen in cultures, RFC can be cultured without disrupting the rosettes with proteolytic enzymes. Such antigen affinity-enriched B cells can be cultured at low cell densities of 1.5-3 x 10^4/ml. The responsiveness of the RFC to LPS or LPS and EL-4 sup, as assessed by proliferation and differentiation into antibody-secreting cells, is the same as spleen cells cultured at a higher density and containing the same number of specific B cells. On an average, 0.3 ± 0.06% of the spleen cells form rosettes. The spleen cells (as recovered above), cultured at 5 x 10^6/ml, generated the same number of CRBC-specific PFC as 1.5 x 10^4 RFC/ml. This is an indication that the purification procedure does not result in the loss of responsive B cells.

The antigen specificity of the RFC deserves some comment. Firstly, spleen cells (Table 2) or T cell-depleted spleen cells (Table 3) cultured in the presence of LPS and EL-4 sup failed to generate an anti-CRBC response in the absence of the antigen (CRBC). Furthermore, this anti-CRBC response generated by spleen cells or T cell-depleted spleen cells in the presence of CRBC is not specific for the stimulating MHC haplotype of CRBC (Tables 2 and 3). This is an expected finding given the fact that the cells were not selected for specific antigen binding. The PFC response of rosettes in the presence of LPS and EL-4 sup, however, is much more specific for the MHC haplotype of the stimulating CRBC (Table 4). Serologically, B^2 shares more public specificities with B^{15}, B^{19}, and B^{21} than with B^{13} and B^{14} (Longenecker *et al*, 1976). Table 4 shows that B^2 RFC generated 60-70% anti-B^2 PFC with some cross-reaction against B^{14}. The generation of an antibody response requires the continuous presence of CRBC. I was unable to derosette RFC and reculture the B cells without loss of the ability to generate PFC. In summary, these observations indicate that rosetting is an effective method to enrich antigen-specific B cells.

EL-4 sup was obtained from the parent EL-4 line as described by Farrar *et al* (1982). The EL-4 cell line can be stimulated by nontoxic concentrations of PMA (10 ng/ml) in the absence of Con A to produce large amounts of factors. The culture supernatant obtained contains a variety of biological activities including TRF, BSF-1, and IL-2 (Farrar *et al*, 1982; Howard *et al*, 1982). The ability of EL-4 sup to induce differentiation of RFC in the presence of LPS was mainly due to the presence of TRF-like activity in the EL-4 sup. The dilution of EL-4 sup used for my studies did not significantly support the growth of HT-2 cells, a bioassay for IL-2 activity, but induced proliferation of anti-IgM-stimulated B cells (co-stimulator assay for BSF activity) to a small but significant degree (Figure 8). The EL-4 sup *per se* had IL-2-like activity at lesser dilutions. EL-4 sup obtained from EL-4 grown in serum-free medium was functionally similar to the EL-4 sup described in these studies. Similary, preparations of EL-4 sup obtained by fractionation over a Sephadex G-100 column were functionally identical to unfractionated EL-4 sup.

Kinetic analysis of time of addition of LPS or EL-4 sup reveals that both LPS and EL-4 sup are required by day 1 of a 4.5 day PFC response (Figure 7). The ability of EL-4 sup to induce differentiation was critically dependent on prior proliferative signals provided by LPS. Two-step cultures (or wash-out experiments) to demonstrate the time period during which LPS or EL-4 sup is required were unsuccessful using RFC. This suggests that removal of growth-promoting activities present in culture supernatant by washing cell cultures irreversibly arrests B cell in a state of partial activation. Importantly, however, the proliferation of RFC induced by LPS was not affected.

In conclusion, LPS by itself induces proliferation of B cells but, in addition, requires T cell lymphokines for differentiation into antibody-secreting cells. In the next section, correlations between early biochemical changes and B cell activation in the context of this model, are reported.

B. Correlation Between Early Changes in Na^+/K^+ ATPase, Amino Acid Transport, and B Cell Activation

LPS and EL-4 Sup Activate $^{86}Rb^+$ Influx

Changes in the rate of transport of monovalent cations across the cell surface membrane in many experimental models have been associated with the triggering of cells to undergo proliferation (Kaplan, 1978; Smith and Rozengurt, 1978; Rozengurt *et al*, 1981; Hesketh *et al*, 1983) and maturation (Bernstein *et al*, 1976; Bridges *et al*, 1981; Rosoff and Cantley, 1983). Activation of the Na^+/K^+ ATPase pump has been reported to be required for cells to proceed through blast formation accompanied by DNA synthesis (Kaplan and Owens, 1982). Since the differentiative signal can only be effective in conjunction with the proliferative signal, it was of interest to search for a correlation between these events and early changes in K^+ influx, as monitored by $^{86}Rb^+$ uptake (Robinson and Flashner, 1979).

As discussed in the previous section, B cells were affinity-enriched for chicken MHC antigens of a given B locus haplotype by the antigen-rosette technique. However, the stimulation of RFC by LPS induced a 30-fold increase in proliferation without the generation of a significant PFC response. The addition of both LPS and maturation factor-containing EL-4 sup triggered the proliferating B cells to differentiate into PFC. In the absence of LPS, the EL-4-derived signal did not induce differentiation or proliferation (Table 7). Marginal proliferation was only induced at excessively high concentrations of EL-4 sup. As discussed elsewhere, activation of Na^+/K^+ ATPase was found to be essential for blast transformation and DNA synthesis. An increase of Na^+/K^+ ATPase activity, in the absence of proliferation, has never been shown under physiological conditions without the introduction of agents that inhibit DNA synthesis. This is especially relevant since such inhibitors may have additional effects that cannot be controlled. Thus, it seemed important to investigate whether EL-4 sup, which itself does not induce proliferation, could cause an increase of $^{86}Rb^+$ influx in RFC. A maximal increase in $^{86}Rb^+$ influx in mouse splenocytes stimulated with Con A was shown to require approximately 14 hr (Kaplan and Owens, 1982). Our data confirmed that $^{86}Rb^+$ influx and proline transport reached maximal levels in murine B lymphocytes after 14 hr of appropriate stimulation. For all assays described, parallel cultures were maintained and the level of thymidine uptake assessed at day 4 for RFC and day 2 for spleen cells.

RFC stimulated with LPS or EL-4 sup for 14 hr were used for the $^{86}Rb^+$ uptake assay. During a 20 min incubation with $^{86}Rb^+$ of RFC preparations that had been purified of unbound CRBC, a linear increase in isotope uptake was observed (Figure 9). Based on this finding, subsequent measurements of $^{86}Rb^+$ influx were done at 1 and 21 min of the assay. It

Table 7

Effect of LPS and EL-4 Sup on the Proliferation and Differentiation of RFC.

Stimuli[1]	^{3}H-Thymidine Incorporation[2] (cpm/culture) ± S.D.	PFC/culture[3]
None	261 ± 64	<1
LPS	5,824 ± 24	8 ± 2
EL-4 Sup	747 ± 21	<1
LPS + EL-4 Sup	6,452 ± 97	1,380 ± 36

[1] RFC were cultured at 3 x 10^3 cells/well in the presence of 10 μg/ml of LPS or 50 μl/ml of EL-4 sup (diluted 1:20).

[2] Cultures were pulsed at 4 days with ^{3}H-thymidine for 4-6 hr and harvested.

[3] Cultures were assayed on day 4.5 for anti-B^2 IgM PFC. Data represent means of triplicate experiments ± S.E.

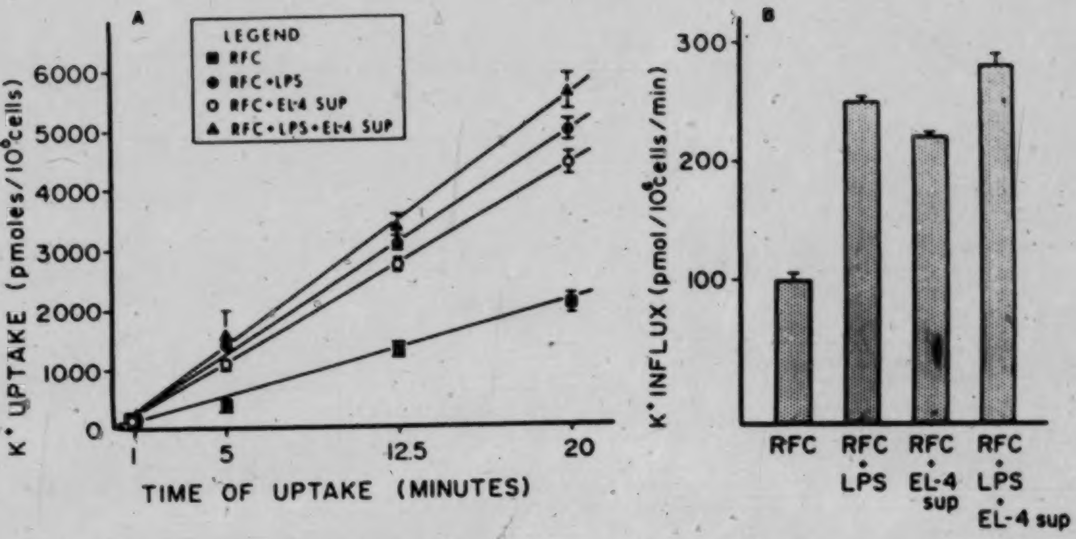

Figure 9. Activation of ^{86}Rb$^+$ influx by LPS and/or EL-4 sup. RFC cultured at 6 x 10^6/ml for 14 hr were centrifuged through Ficoll to remove CRBC and ^{86}Rb$^+$ was added as a substitute tracer for K$^+$. ^{86}Rb$^+$ uptake, expressed in picomoles/10^6 cells (100 pmol/10^6 cells/min = 6 fmol/cell/hr), was measured at 1, 5, 12.5, and 20 min (A) as described in *Materials and Methods*. ^{86}Rb$^+$ influx (B) calculated by linear regression analysis from (A) is expressed in picomoles/10^6 cells/min.

is of particular interest that activation of the Na$^+$/K$^+$ ATPase appeared to be triggered to almost the same degree by both LPS and EL-4 sup (Figure 9). This occurs in spite of the fact that EL-4 sup, in the absence of LPS, causes only minimal proliferation. Table 8 summarizes the ^{86}Rb$^+$ influx obtained from additional experiments. Spleen cells and spleen cells depleted of T cells by treatment with anti-Thy-1.2 and complement, when cultured in the absence of antigen, showed a similar increase in the rate of ^{86}Rb$^+$ influx in response to LPS and/or EL-4 sup (Table 9). Thus activation of Na$^+$/K$^+$ ATPase occurs independently of T cells and antigen.

To ascertain that ^{86}Rb$^+$ influx reflects the function of Na$^+$/K$^+$ ATPase activity, the effect of ouabain on this system was tested. Ouabain specifically inhibits the active translocation of Na$^+$ and K$^+$ across the membrane and prevents lymphocyte transformation (Quastel and Kaplan, 1970). The binding of ouabain to the large subunit of (Na$^+$,K$^+$)-ATPase is reversible and is inhibited by high concentrations of K$^+$ (Schwartz *et al*, 1975). In the presence of 2 mM ouabain, about 70-80% of the total ^{86}Rb$^+$ influx was inhibited (Figure 10). Thus most of the uptake of the isotope in response to LPS or EL-4 sup is dependent on Na$^+$/K$^+$ ATPase activity. Efflux of ^{86}Rb$^+$ from RFC cultures preloaded with the radioisotope for 14 hr was also measured and compared with ^{86}Rb$^+$ influx in the presence of LPS or EL-4 sup. In cultures of RFC stimulated with LPS or EL-4 sup, the percentage increases in ^{86}Rb$^+$ influx and efflux were the same, indicating that increases of ^{86}Rb$^+$ influx in stimulated RFC did not cause a net accumulation of ^{86}Rb$^+$ (data not shown).

The effect of ouabain on LPS-induced RFC proliferation is shown in Figure 11. Ouabain, at a concentration of 0.25-0.5 mM, effectively inhibits proliferation of LPS stimulated RFC regardless of the time of addition of the inhibitor (0, 48, or 72 hr). This effect of ouabain on proliferation can be attributed to its inhibition of Na$^+$/K$^+$ ATPase. The concentrations of ouabain required to inhibit proliferation and that required for inhibition of Na$^+$/K$^+$ ATPase are not the same. This is due to a difference in the length of time of incubation of cells with ouabain for inhibition of both proliferation and Na$^+$/K$^+$ ATPase activity. Parallel experiments measured the effect of ouabain on differentiation of RFC in the presence of LPS and EL-4 sup. The results show that addition of 0.25 to 0.5 mM

Table 8

^{86}Rb^{+} Influx in RFC Stimulated with LPS and/or EL-4 Sup[1]

Cell Culture	Experiment[2]	
	1	2
RFC	106 ± 19	137 ± 7
RFC + LPS	144 ± 6	189 ± 6
RFC + EL-4 Sup	175 ± 14	192 ± 6
RFC + LPS + EL-4 Sup	235 ± 6	ND[3]

[1] After 14 hr of culture, ^{86}Rb^{+} was measured at 1 and 21 min of assay as described in *Materials and Methods*. Results are expressed in pmol/10^6 cells/min ± S.D.

[2] ^{86}Rb^{+} influx is shown in each column from two representative experiments.

[3] Not done.

Table 9

$^{86}Rb^+$ Influx and Proline Transport in Spleen Cells and Thy-1.2-Depleted Spleen Cells

Cells	Treatment[1]	Stimulant	Experiment[2]	
			$^{86}Rb^+$ Influx	Proline Transport
Spleen Cells	Anti-Thy-1.2 and Complement	None	39 ± 3	1.1 ± 0.3
		LPS	91 ± 4	3.5 ± 0.4
		EL-4 Sup	81 ± 3	1.1 ± 0.2
Spleen Cells	None	None	35 ± 5	0.6 ± 0.0
		LPS	88 ± 3	2.1 ± 0.3
		EL-4 Sup	69 ± 2	0.9 ± 0.1

[1] Spleen cells were depleted of T cells as discussed in *Materials and Methods*.

[2] After 14 hr of culture, $^{86}Rb^+$ influx and proline transport were measured as described earlier. Results are expressed in pmol/10^6 cells/min ± S.D.

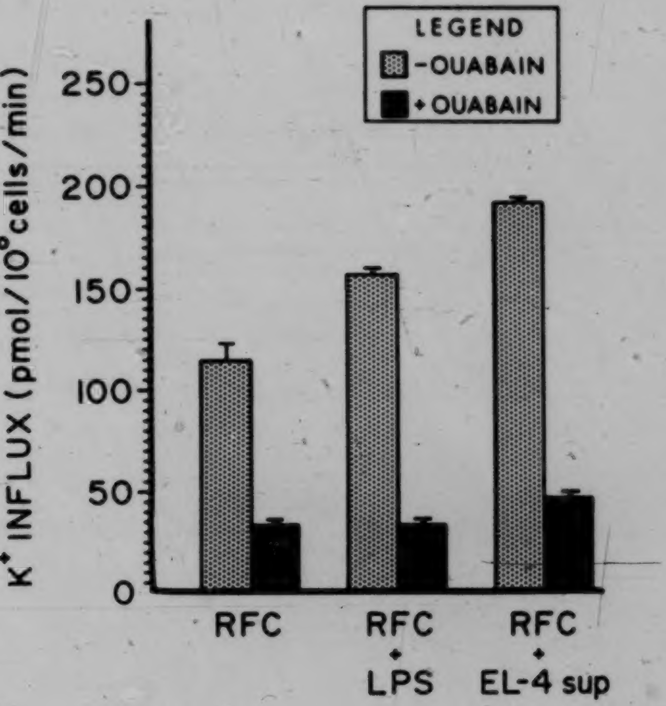

Figure 10. Inhibition of ^{86}Rb$^+$ influx by ouabain. RFC were stimulated with LPS or EL-4 sup for 14 hr and ^{86}Rb$^+$ uptake measured at 1 and 21 min as described in *Materials and Methods*. Alternatively, ^{86}Rb$^+$ uptake was measured in the presence of 2 mM ouabain. ^{86}Rb$^+$ influx is expressed in picomol/10^6 cells/min ± S.D.

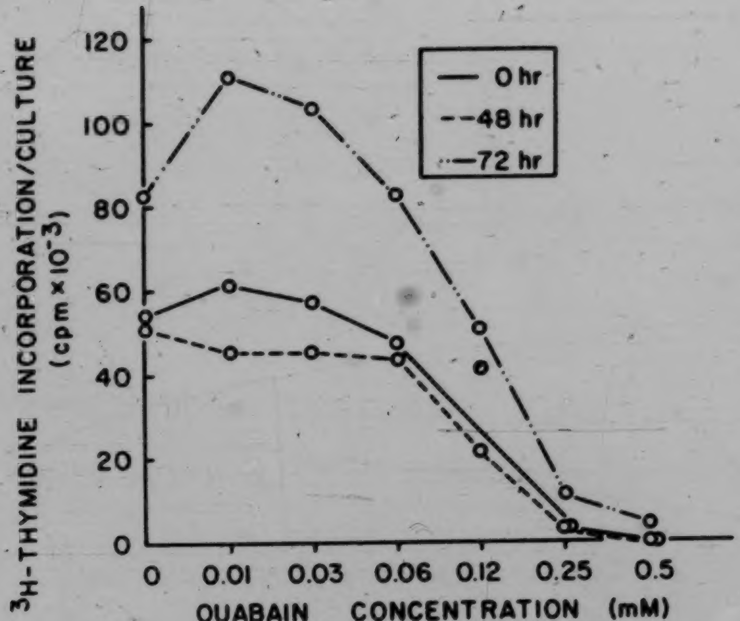

Figure 11. Effect of ouabain on LPS-induced proliferation. RFC (3 x 10^5/well) were cultured in the presence of LPS for 4 days. Doubling dilutions of ouabain were added at zero time, 48, or 72 hr after the initiation of culture. Cultures were pulsed with ^{3}H-thymidine for 4 hr and harvested. Results represent means of triplicate experiments expressed as cpm/culture ± S.D.

ouabain as late as 72 hr following onset of culture, inhibits the PFC response by RFC.

It was concluded that activation of Na^+/K^+ ATPase, known to be linked to proliferation of B lymphocytes (Kaplan and Owens, 1982), is in itself insufficient to act as a stimulus for either proliferation or terminal differentiation. Both LPS and EL-4 sup caused enhancement of $^{86}Rb^+$ influx, while LPS, but not EL-4 sup, triggered significant proliferation of RFC. The increase in Na^+/K^+ ATPase activity induced by LPS or EL-4 sup varied from 1.5-fold to more than 2-fold over values obtained from resting unstimulated B cells. Antigen-containing RFC cultures exhibited greater activity of Na^+/K^+ ATPase than spleen cells. The possibility that binding of CRBC to B cells *per se* may be responsible for this increase cannot be excluded at the present time.

LPS, but Not EL-4 Sup, Activates Proline Transport

It has been shown that transport of neutral amino acids invariably precedes DNA replication after mitogen activation in different cell types (reviewed by Shortwell *et al*, 1983). It was, therefore, of interest to determine whether activation of Na^+/K^+ ATPase is accompanied by an increased rate of proline uptake. Proline transport in maximally stimulated RFC during a 5 min pulse with the tritiated amino acid was linear (data not shown); therefore, in subsequent studies, proline uptake was assayed at zero time and after 5 min following addition of ^3H-proline to cultures. RFC cultured for 14 hr in the presence of LPS or LPS and EL-4 sup responded with a similar and significant increase in the rate of proline transport relative to unstimulated control cultures. Interestingly, EL-4 sup, which was shown to activate the Na^+/K^+ ATPase system, failed to stimulate proline transport (Figure 12). Addition of EL-4 sup to LPS-stimulated cells did not further increase the rate of amino acid transport induced by the mitogen. It is important to note from Figure 12 that transport of proline occurs *via* a Na^+-dependent amino acid transport system since 70-80% of proline uptake was inhibited by the withdrawal of extracellular Na^+. Table 10 provides additional data from experiments on proline transport in RFC. When cultured in the absence of antigen, spleen cells and spleen cells depleted of T cells by anti-Thy-1.2 and complement showed a similar increase in the rate of proline influx in response to LPS; however, EL-4 sup

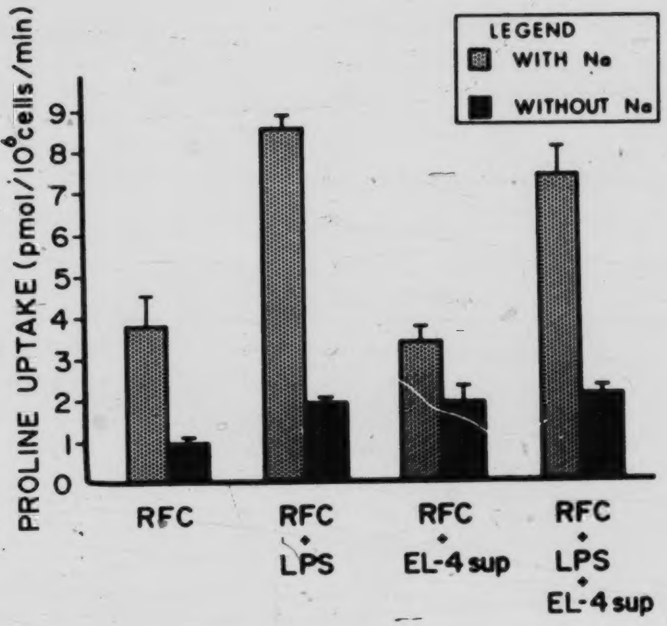

Figure 12. Rate of proline transport in RFC stimulated with LPS and/or EL-4 sup. Proline uptake was measured at zero time and at 5 min of the assay in the presence or absence of Na^+ as described in *Materials and Methods*. Results are expressed in picomoles/10^6 cells/min ± S.D.

Table 10

Proline Transport in RFC Stimulated with LPS and/or EL-4 Sup[1]

Cell Culture	Experiment[2]	
	1	2
RFC	1.3 ± 0.15	3.36 ± 0.6
RFC + LPS	6.2 ± 0.49	6.46 ± 1.0
RFC + EL-4 Sup	2.3 ± 0.19	4.33 ± 0.8
RFC + LPS + EL-4 Sup	ND[3]	4.44 ± 0.3

[1] After 14 hr of culture, proline transport was measured at zero time and at 5 min of assay as described in *Materials and Methods*. Results are expressed in pmol/10^6 cells/min ± S.D.

[2] Proline transport from two representative experiments is shown in each column.

[3] Not done.

failed to stimulate proline transport (Table 9).

LPS, but Not EL-4 Sup, Induces RNA, Protein, and DNA Synthesis in RFC

In our experimental models, EL-4 induced B cells to mature into antibody-secreting cells provided they had been activated by LPS (Table 7). In the absence of LPS, EL-4 sup failed to induce an antibody response, although it readily activated the Na^+/K^+ ATPase pump which is required for subsequent entry into S phase. In view of this unexpected finding it was important to ascertain in a time course study whether EL-4 sup, in the absence of LPS, initiated RNA and protein synthesis. ^{3}H-uridine was used as a tracer for RNA accumulation. In the presence of LPS or LPS and EL-4 sup, the incorporation of ^{3}H-uridine became evident by 20 hr of culture and continued to increase steadily up to 88 hr (Figure 13A). Incorporation of ^{3}H-leucine and ^{3}H-thymidine as tracers of protein accumulation and DNA synthesis, respectively, showed a steady increase from 40 to 88 hr, followed by a sharp decline at 110 hr of culture (Figures 13B,13C). In the presence of EL-4 sup alone, incorporation of ^{3}H-thymidine, ^{3}H-uridine, and ^{3}H-leucine, was minimal. EL-4 sup did not induce RNA, protein, or DNA synthesis in cells stimulated with LPS. It is clear from these results that LPS plays a critical role in inducing RFC to enter S phase of the cell cycle as marked by RNA, protein, and DNA synthesis. The percentage increases in viable cell yield in cultures stimulated with LPS or with LPS and EL-4 sup for 88 hr of stimulation were 390 percent and 280 percent, respectively (Figure 14); the value correlates with the one obtained from measurements of ^{3}H-thymidine incorporation.

EL-4 Sup Induces an Increase in Cytoplasmic μ mRNA, PFC, and IgM Secretion of LPS-Stimulated RFC

Within the context of this study on early functional parameters of B lymphocyte activation, the presence of cytoplasmic mRNA encoding μ chain of IgM was detected using a ^{32}P-labelled probe, p104Eμ12, which hybridizes to both secretory and membrane forms of IgM (Calame *et al* 1980). The recombinant plasmid, p104μ12 (pμ12), contains a cDNA sequence from the H chain mRNA of the IgM-producing myeloma tumour, M104E (Calame *et al*,

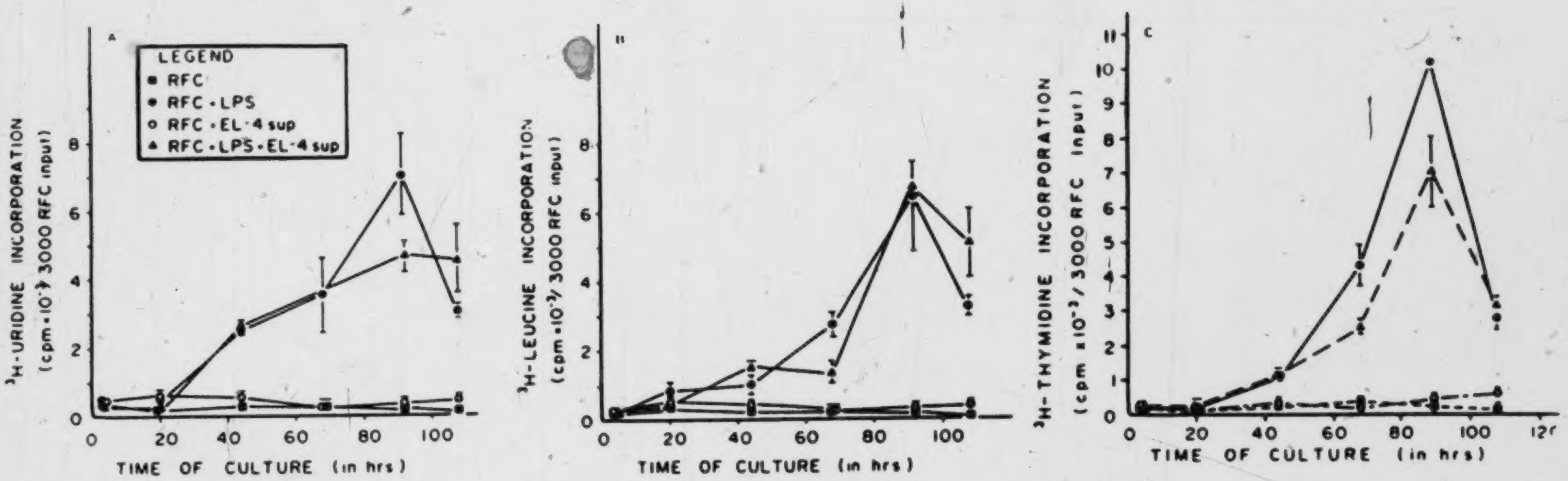

Figure 13. Kinetics of RNA, protein, and DNA synthesis in RFC activated with LPS, EL-4 sup, or LPS and EL-4 sup. RFC were cultured as described earlier from 4 to 110 hr and were assayed for incorporation of ^{3}H-uridine (A), ^{3}H-leucine (B), and ^{3}H-thymidine (C).

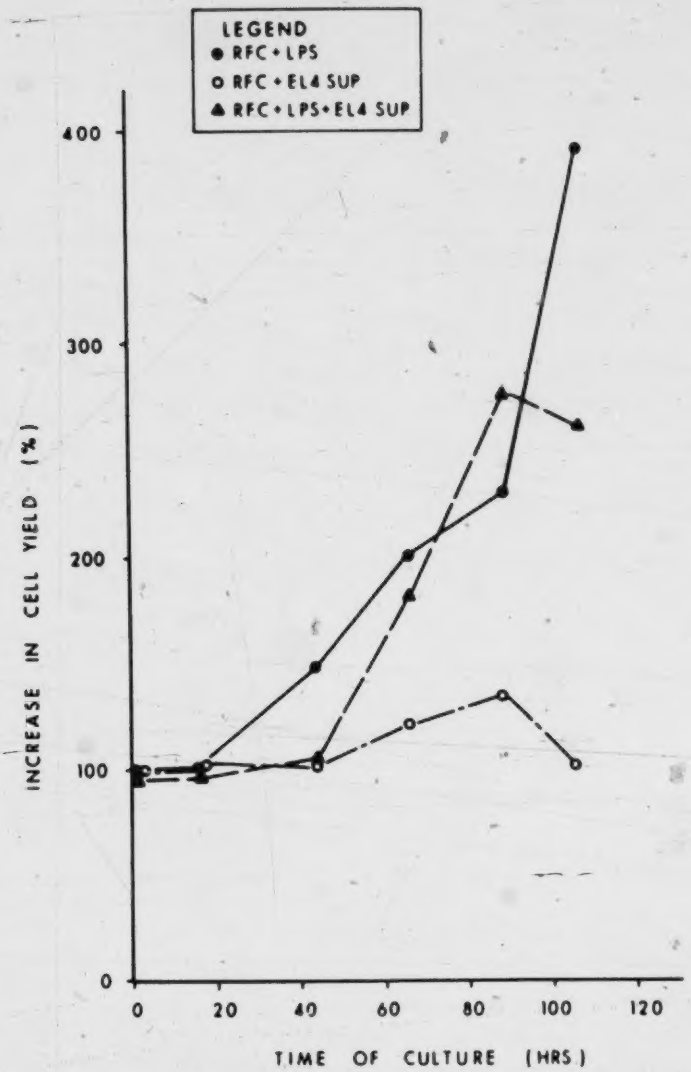

Figure 14. Percentage increase in cell yield of RFC stimulated with LPS, EL-4 sup, or LPS and EL-4 sup. RFC were cultured for 4.5 days and cells counted at several time intervals by eosin exclusion. At each time point, viability of RFC without stimulants was considered as 100%.

1980). The double-stranded cDNA segment was originally inserted into the *Pst* site of pBR322 by a poly(dC:dG) tailing procedure which reconstituted the *Pst* site at each side. Comparison of the DNA sequences with the protein sequences of the M104E myeloma μ chain indicated that pμ12 contained Cμ coding sequences extending from residue 300 to the C-terminus of the μ chain at position 576. An IgM- and IgG$_{2a}$-secreting myeloma, Y5781.4 and X5563.2, respectively (kindly provided by Dr. B.M. Longenecker, Department of Immunology, University of Alberta), were used as positive and negative controls for the dot-blot experiments. As indicated in Figure 15, X5563.2 cells contained no RNA complementary to the μ cDNA probe. However, RNA from Y5781.4, an IgM-secreting myeloma, hybridized maximally to the μ cDNA probe. As expected, stimulation of RFC with LPS- and EL-4 sup induced μ chain-encoding mRNA (Figure 15). The kinetics of the increase of μ mRNA upon addition of LPS or LPS and EL-4 sup revealed the faint appearance of hybridization to the μ probe at 60 hr of culture. This increase in μ mRNA reached a maximum at 88 hr of culture (Figure 16). Although LPS did not induce a PFC response, it, nevertheless, induced an increase in μ mRNA, albeit 2- to 8-fold less than that induced by LPS and EL-4 sup. The significance of μ chain-specific mRNA induced by LPS is not clear. RFC cultured in the presence of EL-4 sup, which was shown in the preceeding section to activate Na$^+$/K$^+$, showed a small but significant increase μ mRNA.

The μ probe used in these experiments hybridizes to a stretch of nucleic acids common to both μ_m and μ_s mRNA. To further quantitate the amounts of μ_m and μ_s in RFC stimulated with LPS or LPS and EL-4 sup, RNA from these cells was analyzed by northern gels. The μ_m and μ_s forms of μ mRNA are 2.7 and 2.4 kb long, respectively. Analysis of the RNA from RFC stimulated with LPS or LPS and EL-4 sup on gel and hybridization to the μ probe is shown in Figure 17. The quantitative difference in μ_m and μ_s RNA in RFC stimulated with LPS or LPS and EL-4 sup was difficult to assess.

Next, antigen-specific and -nonspecific IgM secreted into the supernatants of LPS-stimulated RFC cultures, were quantitated. This was done to critically assess whether the μ mRNA induced in RFC by LPS was later secreted as IgM although no antigen-specific PFC response was obtained. In the presence of LPS and EL-4 sup, an optimal increase in

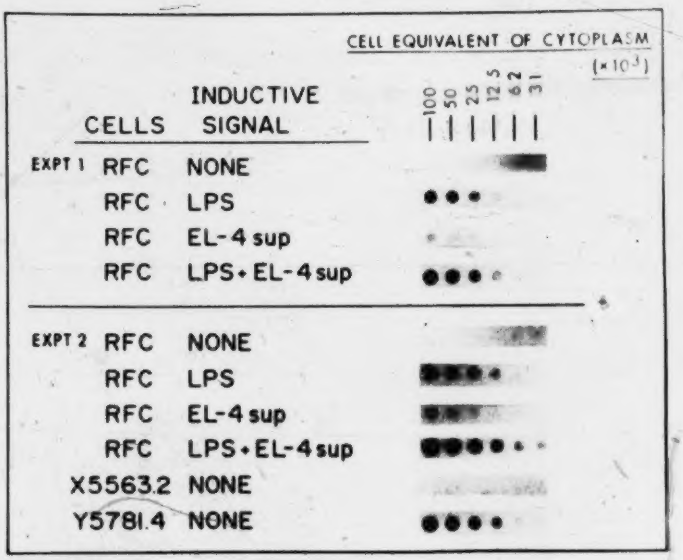
CELL EQUIVALENT OF CYTOPLASM
(×10³)
100
50
25
12.5
6.2
3.1
CELLS
INDUCTIVE SIGNAL
EXPT 1
RFC NONE
RFC LPS
RFC EL-4 sup
RFC LPS+EL-4 sup
EXPT 2
RFC NONE
RFC LPS
RFC EL-4 sup
RFC LPS+EL-4 sup
X5563.2 NONE
Y5781.4 NONE

Figure 15. Dot-blot analysis of cytoplasmic μ mRNA. RFC were cultured as described in *Materials and Methods* with LPS, EL-4 sup, or LPS and EL-4 sup. After 86 hr, cytoplasmic RNA was prepared from cells and hybridized with a ^{32}P-labelled μ probe (pl04E$_{\mu}$12).

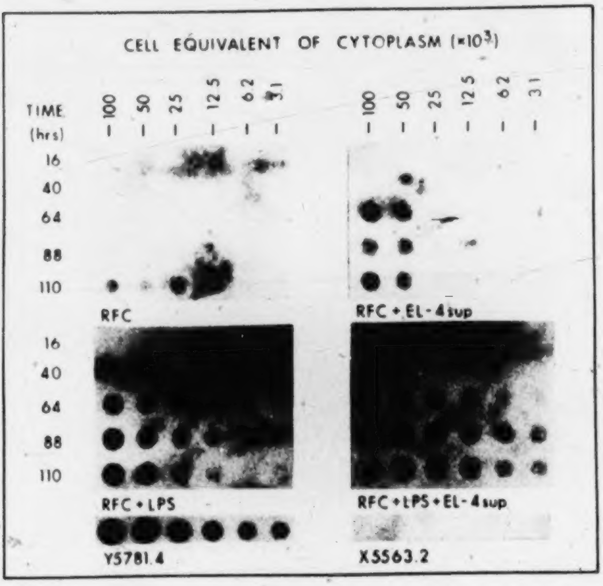
CELL EQUIVALENT OF CYTOPLASM (×10³)
TIME (hrs)
100
50
25
12.5
6.2
3.1
16
40
64
88
110
RFC
RFC + EL-4 sup
RFC + LPS
RFC + LPS + EL-4 sup
Y5781.4
X5563.2

Figure 16. Kinetics of μ mRNA induction. RFC were cultured as described in *Materials and Methods* with LPS, EL-4 sup, or LPS and EL-4 sup. At 16, 40, 64, 88, and 110 hr of culture, cytoplasmic RNA was prepared from cells and hybridized with a ^{32}P-labelled μ probe (p104E μ12).

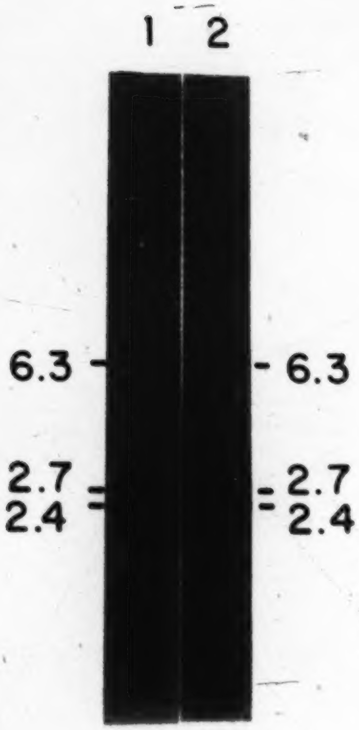

Figure 17. Northern analysis of μ mRNA from RFC activated with LPS and EL-4 sup. RNA (20 μg), extracted from LPS- (lane 1) or LPS and EL-4 sup- (lane 2) stimulated RFC, was electrophoresed on a 1% formaldehyde agarose gel and transferred to nitrocellulose paper as described in *Materials and Methods*. The northern blot was hybridized to ^{32}P-labelled μ cDNA probe (6.4 x 10^{8} cpm/μg) and exposed to X-ray film. Molecular weights (indicated in kb) were determined by extrapolation of the position of ribosomal RNA bands visualized by ethidium bromide staining of gel (28S=6.3 kb, 18S= 2.36 kb).

IgM secretion (Figure 18) and numbers of PFC (Figure 19) was observed at 110 hr and 86 hr, respectively. In the presence of LPS or EL-4 sup alone, there was no increase in B^2-specific IgM PFC (Figure 19). There was a small increase in IgM secreted by RFC stimulated with LPS alone (Figure 18). This was an unexpected finding since RFC stimulated with LPS alone did not generate any B^2-specific IgM PFC. The increase in total IgM and antigen-specific IgM PFC compares favorably with an increase in μ RNA accumulation (Figure 15). EL-4 sup did not increase the amount of μ chain-encoding mRNA synthesis, IgM secretion or PFC to any degree comparable to that induced by LPS and ~~EL-4~~. This increase in amount of μ chain-specific mRNA induced by LPS was to a lesser amount than that induced by LPS and EL-4 sup. Since only a small amount of non-CRBC-specific antibody was detected in cultures stimulated by LPS alone (Figure 18), it is possible that the μ mRNA observed is due to non-CRBC-specific contaminating B cells. This possibility is discussed later in this section. Time kinetics of IgG secretion indicated that there was little IgG secreted by RFC upon stimulation with LPS and EL-4 sup at day 4 (Table 11). Spleen cells and spleen cells depleted of T cells by treatment with anti-Thy-1.2, when cultured in the absence of antigen, showed an increase in total IgM secretion in the response to LPS or LPS and EL-4 sup (Table 12). The amount of IgM secreted in the presence of LPS was higher than in the presence of LPS and EL-4 sup. The reason for this difference is not known. Thus, induction of total IgM secretion occurs independently of T cells or antigen in spleen cells depleted of T cells. As shown earlier (Table 3), there are no B^2-specific PFC induced in the absence of antigen.

Effect of EL-4 Sup is Not Due to Contamination by PMA

PE induce depolarization of membranes (Monroe and Cambier, 1983) and increase of Ia expression on lymphocytes. However, PE have been shown not to trigger B cells to enter the G_1 phase of the cell cycle (Thompson *et al*, 1985). The possibility that in our experiments, contamination of EL-4 sup with PMA was responsible for the observed increase in $^{86}\text{Rb}^+$ influx, can be ruled out for the following reasons. Assuming that all of the PMA was retained despite extensive dialysis of the ammonium sulphate precipitate of EL-4 sup, not more than 0.25 ng/ml of PMA could have been present in our cultures. Lindsten *et al* (1984)

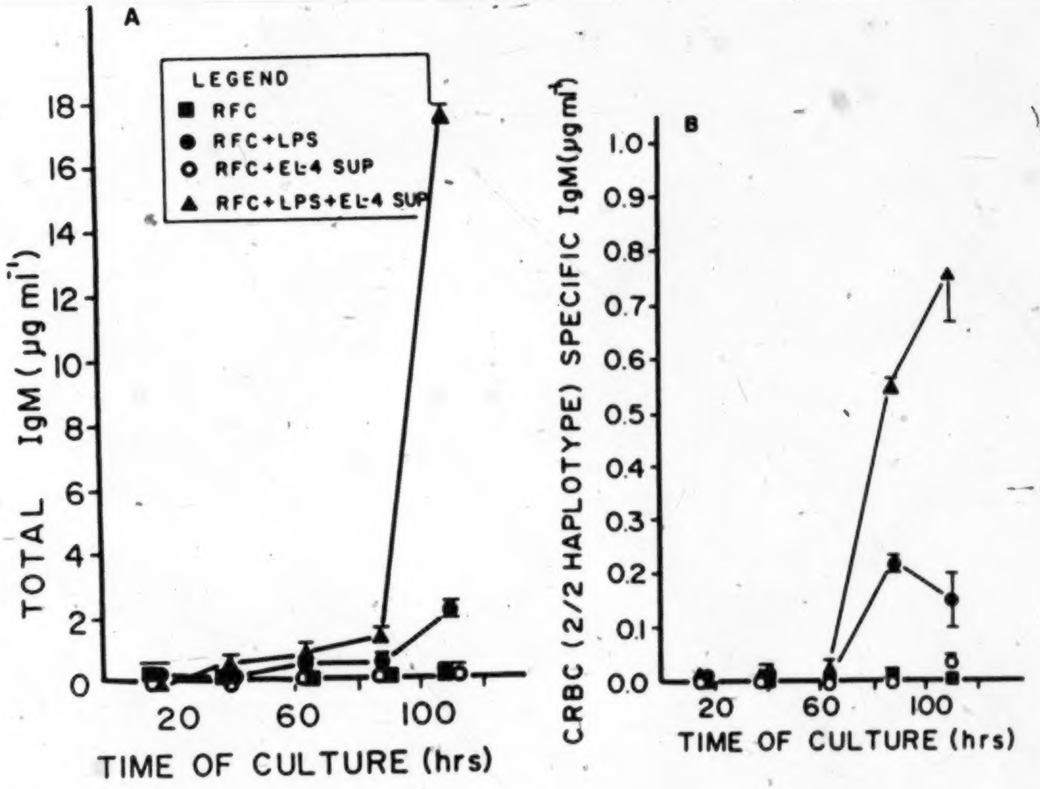

Figure 18. Kinetics of total and B^1-specific secretory IgM. Culture supernatants were collected at several time intervals from 16 to 110 hr of culture. The amount of total IgM was estimated by RIA. The lower limit of detection of IgM was 0.025 μg/ml. Results are expressed as μg/ml ± S.D.

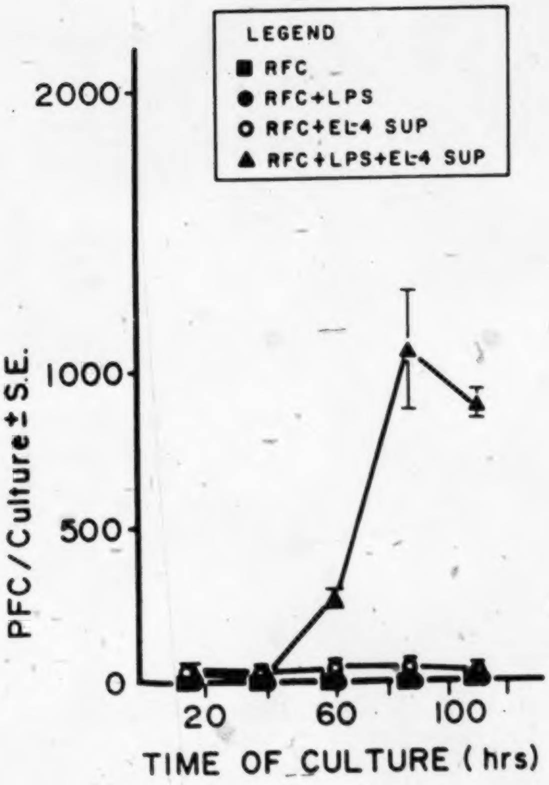

Figure 19. Kinetics of B^1-specific IgM-secreting cells. Cultures of RFC stimulated with LPS, EL-4 sup, or LPS and EL-4 sup were assayed for B^1-CRBC-specific IgM PFC from 16 to 110 hr. Results represent means of triplicate experiments expressed as PFC/culture ± S.E.

Table 11

Kinetics of IgG Secretion by RFC

Stimuli[1]	IgG-Secreted ($\mu g/ml$)[2]				
	16 hr	40 hr	64 hr	88 hr	110 hr
None	<0.01	<0.01	<0.01	<0.01	<0.01
LPS	<0.01	<0.01	<0.01	0.012 ± 0.001	0.120 ± 0.001
EL-4 Sup	<0.01	<0.01	<0.01	<0.01	0.008 ± 0.002
LPS + EL-4 Sup	<0.01	<0.01	<0.01	0.096 ± 0.004	0.270 ± 0.040

[1] RFC were cultured at 3×10^3 cells/well with various stimuli.

[2] At various times of culture, culture supernatants were collected and assayed for IgG secretion by RIA as described in *Materials and Methods*. A twelve-point standard curve was obtained with the experiment. The lower limit of IgM detection was 10^{-4} $\mu g/ml$. Results are expressed in $\mu g/ml$ of culture ± S.D.

Table 12

Spleen Cells and Thy-1.2-Depleted Spleen Cells Secrete IgM on Stimulation with LPS

Cells	Stimuli[1]	IgM-Secreted (µg/ml)[2]	
		Total IgM	Anti-B^3 IgM
Spleen Cells	None	2.90 ± 0.50	<0.01
	LPS	19.30 ± 3.21	<0.01
	EL-4 Sup	1.43 ± 0.27	<0.01
	LPS + EL-4 Sup	12.10 ± 2.50	<0.01
Thy-1.2-Depleted Spleen Cells	None	$<10^{-3}$	<0.01
	LPS	46.10 ± 7.81	<0.01
	EL-4 Sup	1.08 ± 0.42	<0.01
	LPS + EL-4 Sup	17.30 ± 3.50	<0.01

[1] Spleen cells and Thy-1.2-depleted spleen cells were cultured at 2 x 10^5 cells/well and 2 x 10^4 cells/well, respectively, in the absence of antigen.

[2] Culture supernatants were collected at 4.5 days. The amount of total and anti-B^3 IgM was estimated by RIA. Lower limit of detection for total and anti-B^3 IgM was 1 x 10^{-4} and 0.01 µg/ml, respectively.

reported that PMA at a final concentration of 10 ng/ml had proven optimal in inducing changes in murine B cell surface markers (sIgM, sIgD, sIa). The increase in $^{86}Rb^+$ influx stimulated by PMA at concentrations of 10, 1, and 0.1 ng/ml were 85, 62, and 44 pmoles/10^6 cells/min, respectively, in the absence of LPS. In the same experiment, LPS-stimulated cells had a $^{86}Rb^+$ influx of 87 pmoles/10^6 cells/min.

The effect of PMA on differentiation of RFC is shown in Table 13. The combination of LPS and PMA could not induce a PFC response in RFC even at high concentrations (100 ng/ml). In contrast, as little as 0.1 ng/ml of PMA was inhibitory for the PFC response. PMA at a concentration of 10 ng/ml or higher stimulated proliferation of RFC in the absence of LPS and also enhanced the proliferation of RFC stimulated with LPS (Figure 20). These results rule out the possibility that a PMA contaminant in the EL-4 sup may be involved in early steps of B cell activation since not more than 0.25 ng/ml of PMA could be present in our cultures. The fact that very low concentrations of PMA are inhibitory for the PFC response also argues against the possibility.

Discussion

The cellular Na^+ and K^+ concentrations in lymphocytes are maintained by a system of leak fluxes and pumps common to all nucleated cells in which plasma membrane Na^+/K^+-activated ATPase transduces the energy required for uphill ion movements against their respective gradients. The high K^+, low Na^+ environment within the cell provides a mechanism for cell volume regulation, for the transport of substrates dependent on an ionic gradient for accumulation, and an appropriate ionic environment for protein synthesis.

An increase in K^+ influx (as monitored by $^{86}Rb^+$) in murine splenocytes stimulated with Con A requires 6 to 8 hr and is maximal at 14 hr of stimulation (Kaplan and Owens, 1982), whereas, in human peripheral blood lymphocytes, it becomes evident within a few minutes of stimulation (Owens and Kaplan, 1980). Similarly, in the present study, $^{86}Rb^+$ influx and proline transport achieved maximal levels in murine B lymphocytes after 14 hr of stimulation by LPS or EL-4 sup. The reason why an increase in K^+ flux in murine splenocytes takes 14 hr instead of just a few minutes, is unclear. Rosoff and Cantley (1983)

Table 13

Inhibition of PFC Response by PMA

Stimulant[1]	PMA Concentration (ng/ml)[2]				
	0	0.1	1	10	100
None	<1	<1	<1	<1	<1
LPS	<1	14 ± 5	<1	<1	<1
LPS ± EL-4 Sup	1,888 ± 314	22 ± 14	24 ± 20	32 ± 16	<1

[1] RFC (3 x 10^3 cells/well) were cultured in the presence of 10 μg/ml of LPS or 50 μl/ml of EL-4 sup (1:20 dilution).

[2] Various concentrations of PMA were added at the onset of culture. Cultures were assayed for anti-B^1 IgM PFC at the end of 4.5 days. Data represent means of triplicate cultures and results are expressed as PFC/culture ± S.E.

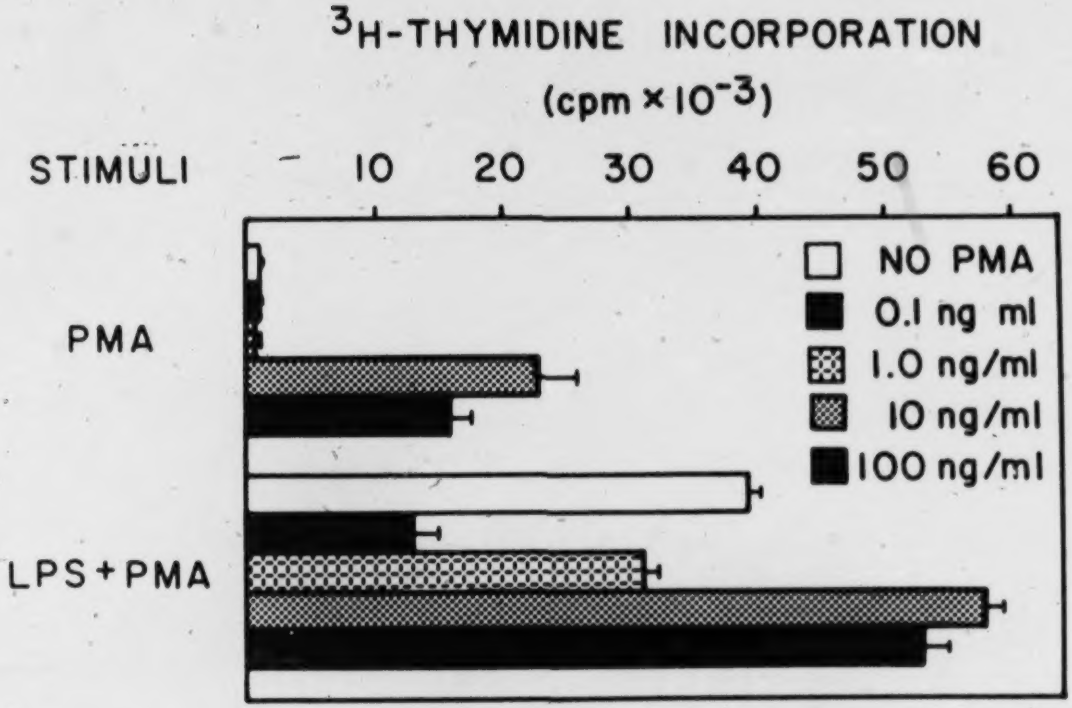

Figure 20. Effect of PMA on proliferation of RFC. RFC (3 x 10^5/well) were cultured in the presence or absence of LPS (10 μg/ml) and various concentrations of PMA were added at the onset of cultures. Cells were pulsed with ^{3}H-thymidine for 4 hr on day 4 and harvested. Data represent means of triplicate cultures expressed as cpm/culture ± S.D.

reported stimulation of ouabain-sensitive ^{86}Rb$^+$ uptake by LPS in the murine pre-B lymphocyte cell line 70Z/3 within 45 min of stimulation. This may have been due to the leukemic nature of the cells. No reports on measurements of K$^+$ influx in purified murine B cells are available for comparison. The activation of Na$^+$/K$^+$ ATPase activity appeared to be triggered to nearly equal degrees by LPS or by EL-4 sup (Figures 9,10, Table 8). Stimulation of RFC with EL-4 sup alone enhanced the ^{86}Rb$^+$ influx but did not increase the transport of proline (Figure 12, Table 10) or the synthesis of RNA, protein, or DNA (Figure 13). It was therefore surprising that in the presence of EL-4 sup only, RFC underwent activation of Na$^+$/K$^+$ ATPase to the same degree as RFC stimulated with LPS. It is well established that the proliferative capacity of both lectin-stimulated and spontaneously-dividing lymphocytes is absolutely dependent upon Na$^+$, K$^+$ transport activity. The cardiotonic steroid, ouabain, specifically, competitively, and reversibly inhibits Na$^+$/K$^+$ ATPase and all parameters of the stimulated state (Quastel and Kaplan, 1968, 1970). Along the same lines, ouabain inhibited the increase in ^{86}Rb$^+$ influx by 70-80% (Figure 10) and also effectively blocked the entry of B cells into S phase of the cell cycle (Figure 11). High concentrations of drugs such as oligomycin inhibit Na$^+$/K$^+$ ATPase and DNA synthesis of stimulated cells but not blast transformation itself (Quastel and Kaplan, 1970). Valinomycin was shown to be much more selective in its action, inhibiting DNA synthesis at concentrations at which RNA synthesis was insensitive. These studies lead to the speculation that activation Na$^+$/K$^+$ may be necessary but insufficient to induce proliferation. The experiments reported here demonstrate, for the first time, stimulation by lymphokines of Na$^+$/K$^+$ ATPase without subsequent induction of DNA synthesis. This is unlike experiments where the activation of Na$^+$/K$^+$ ATPase could be dissociated from proliferation by use of inhibitors.

The increase in K$^+$ influx upon stimulation of RFC with LPS or EL-4 sup results from an enhanced uptake by Na$^+$/K$^+$ ATPase rather than from a decreased efflux. This was indicated by measurements of the ^{86}Rb$^+$ influx and efflux. The results showed that both efflux (58% and 51%) and influx (40% and 55%) were similar in RFC stimulated with either LPS or EL-4 sup. These results are in agreement with Segel and Lichtman (1976) and Kaplan (1978) who showed that the increased K$^+$ influx is balanced by an increased efflux of

K^+. The mechanism of increase in K^+ influx however remains a subject of controversy. Quastel and Kaplan (see Kaplan and Owens, 1980) observed that the V_{max} and not the k_m of transport was changed upon stimulation, leading these investigators to propose that the increase is caused by the appearance of new pump sites. Avendunk and Lauf (1975) attributed the increase in ion transport to an enhanced function of each pump site. The controversy is still unresolved.

The precise regulation of growth and differentiation mediated by cation transport across the membrane is not clearly understood. What is well known is that agents that induce differentiation of cultured cells by causing changes in Na^+, K^+, Ca^{2+}, and H^+ fluxes across the plasma membrane can be replaced by ionophores that transport Na^+, H^+, and Ca^{2+}. A possible connection between the PKC activation cascade and stimulation of Na^+/H^+ exchanges has been proposed by Moolenaar *et al* (1984). A simplistic view would be that activation of PKC stimulates Na^+/H^+ exchanges which lead to an increase in intracellular pH. This increase in $[Na^+]$ probably activates the Na^+/K^+ pump.

RFC stimulated to enter the cell cycle induced by LPS show an increase in the uptake of proline. This increase in transport of proline up to 100-200 percent was found to be dependent on Na^+ (Figure 12, Table 10). Withdrawal of extracellular Na^+, inhibited that increase in proline transport in the presence of LPS by 60-70 percent. Both LPS or LPS and EL-4 sup induced an increase in proline transport as well of RNA, protein, and DNA synthesis (Figure 13). RFC stimulated with EL-4 sup failed to enhance the transport of proline. This was not surprising in view of the finding that EL-4 sup does not induce RFC to proliferate. There is, thus, a stronger correlation between enhanced amino acid transport and progression into cell cycle. In fact, depriving lymphocytes and other cells of amino acids is reported to inhibit cell growth. Bayer *et al* (1980) used nonsteroidal anti-inflammatory drugs to reversibly arrest cell growth of rat HTC cell cultures in the G_1 phase. This inhibition was thought to be due to decreased entry of amino acids by the A system. Decreased transport of amino acids is also the key functional defect in chronic leukemia of B lymphocytes (B cell CLL) (Segel *et al*, 1983).

The increase in amino acid transport induced in rodent lymphocytes by Con A or PHA was found to be sensitive to cycloheximide and puromycin and thus appeared to be dependent on protein synthesis (Van den Berg and Betel, 1974). It is not clear if the same situation exists in human lymphocytes. Data on the inhibition of amino acid transport by protein synthesis inhibitors (Segel and Lichtman, 1979) contrast with reports by others (Mendelsohn *et al*, 1971).

The increase in proline transport in RFC stimulated with LPS or LPS and EL-4 sup was measurable only 14 hr after stimulation. Segel and Lichtman (1980) showed that in human lymphocytes an increase in transport of amino acids may occur as early as 4 hr after onset of culture in the presence of PHA. On the other hand, Borghetti *et al* (1979) showed that enhanced transport of amino acids after PHA treatment of porcine lymphocytes required 24 hr of stimulation. PHA-induced increases in amino acid transport by the L system (see p. 27) were reported to occur within a 16-24 hr exposure to lectin. Similar studies using murine B cells are not available for comparison with the data reported here. The discrepancies in lag times observed with respect to increases in amino acid transport following stimulation of human and mouse lymphocytes are paralleled by the highly variable data on $^{86}Rb^+$ influx.

There was always a small inhibition of proline transport (Table 10) and incorporation of radiolabelled precursors (Table 13) in RFC on stimulation with both LPS and EL-4 sup as compared to LPS alone. The reason for this difference is unclear.

The studies reported here demonstrate that stimulation of RFC with LPS or LPS and EL-4 induces a striking increase in the quantity of μ mRNA. The addition of LPS together with EL-4 sup to RFC consistently induced the synthesis of more μ mRNA than did LPS alone. The effect of B cell stimulation by LPS and T cell-derived factors on Ig gene regulation has been extensively studied at the molecular level. LPS was shown to increase steady-state levels and transcription of μ genes in normal splenic B cells (Lamson and Koshland, 1984; Yuan and Tucker, 1984a,b). Interestingly, LPS does not cause expected increases in μ mRNA levels in BCL-1, presumably because μ gene transcription is already activated in these cells. In BCL-1, LPS treatment results in a 2- to 3-fold increase in μ_s over

μ_m polypeptide, although the levels of transcription of μ mRNA remain unchanged (Yuan and Tucker, 1982). This clearly points to post-transcriptional effects of LPS treatment on Ig gene expression. Like LPS, EL-4 sup was also found to induce μ mRNA synthesis. Vitetta *et al* (1984) reported that EL-4 sup caused an increase in secretion of IgM by BCL-1 cells, which was found to correlate with an increase in μ mRNA. Similarly, a 5- to 10-fold increase in μ mRNA as well as an increase in μ_s over μ_m mRNA was observed by Nakanishi *et al* (1984) on addition of B151-TRF and EL-TRF (T cell-derived factors) to anti-μ and BSF-1-stimulated B cells.

The presence of μ mRNA in LPS-stimulated RFC was surprising because these cultures did not generate any IgM PFC in spite of the significant increase in IgM secretion (Figure 17). In fact, amounts of secretory IgM were found to correlate with levels of μ mRNA in each case tested (Figure 18). It is difficult to account for the IgM secreted by RFC stimulated with LPS in the absence of a PFC response.

It is possible that there is a subset of B cells that secrete IgM upon stimulation with LPS and in the absence of T cell-derived signals. The generation of an IgM PFC response only in the presence of LPS and EL-4 sup is probably due to the ability of EL-4 sup to induce J chain expression. The secretion of the pentameric form of IgM, which is more efficient in binding complement, results in plaque formation. To this end, it has recently been shown that IL-2 induces J chain expression in the murine B cell line BCL_1 and stimulates secretion of pentamers of IgM (Blackman *et al*, 1986).

The possibility that the contamination of EL-4 sup by PMA was responsible for the observed increase of $^{86}Rb^+$ influx or its ability to generate PFC in the presence of LPS deserves some discussion. PMA at the dose expected from contamination (<0.25 ng/ml) is unable to increase $^{86}Rb^+$ influx. The optimal dose of PMA required for proliferation of RFC in the absence of LPS, was shown to be 10 ng/ml, in agreement with previous findings by other investigators (Lindsten *et al*, 1984) (Figure 20). As little as 0.1 ng/ml PMA was sufficient to inhibit the generation of PFC by RFC stimulated with LPS and EL-4 sup (Table 13). Thus, the contamination of EL-4 sup with PMA must be less than 0.1 ng/ml in order to obtain any PFC response. PMA by itself or in combination with LPS was unable to induce any PFC response.

In this section, the biological relevance of early changes in biochemical events with respect to B cell proliferation and maturation, was examined. These experiments have demonstrated the induction of an early event in lymphocyte activation, namely the increase in Na^+/K^+ ATPase, which was shown to be insufficient to bring about DNA synthesis.

C. Role of Recombinant IL-2 and IL-1 in LPS-Dependent Activation of B Cells

The proliferation and differentiation of B cells into antibody-secreting cells is regulated by antigen and cytokines derived from T cells and monocytes. A number of B cell activation models have been described. Upon reviewing the interpretations of these models, it appears that no consensus has emerged concerning the identity of the cytokines believed to control proliferation and antibody synthesis of B cells. IL-2 is reported to function both as a growth and differentiation factor for antigen-specific B cells (Pike *et al*, 1984) as well as B cells stimulated with anti-IgM (Hashimoto *et al*, 1986; Zubler *et al*, 1984; Nakanishi *et al*, 1984). In contrast, *in vivo* antigen-primed B cells have been reported not to require IL-2 to undergo differentiation into antibody-forming cells (Takatsu *et al*, 1985). Other reports suggest that IL-2 has no direct effect on B cells, but acts on contaminating T cells which in turn release B cell-activating factors (Leanderson and Julius, 1986). A similar controversy addresses the role of IL-1 in B cell differentiation (Pike and Nossal, 1985; Howard and Paul, 1983; Chiplunkar *et al*, 1986; Hoffmann, 1980; Booth and Watson, 1984; Leibson *et al*, 1982).

Contradictory statements concerning the requirement for cytokines in B cell activation are possibly due to the different experimental models used by different investigators, many of which may have been insufficiently standardized with respect to B cell heterogeneity, contamination of B cells with other cell types, and definition of cytokines.

The model of B cell activation described in the earlier section requires distinct proliferative (i.e., LPS) and differentiative (i.e., EL-4) signals. For these studies, preparations of rosettes containing very few contaminating nonrosettes were obtained by decreasing the time of centrifugation through Ficoll to 4 min (as described in ***Materials and Methods***). These rosettes are referred to as RFC 4' to distinguish them from the cell preparation described in the preceeding studies, which will be referred to as RFC 25'. The average yield of RFC 4' from spleen cells is 0.64 percent and ninety nine percent of the rosettes consist of small B cells as determined by velocity sedimentation. Recombinant human IL 1 has been used in the following experiments unless otherwise mentioned.

Functional Analysis of RFC 4'

RFC 4' stimulated for 14 hr were spun through Ficoll to remove CRBC. $^{86}Rb^+$ influx was measured at 1 and 21 min of the assay (Table 14). As was the case with less pure RFC 25' reported in the previous sections, activation of $^{86}Rb^+$ influx by LPS or by EL-4 sup was observed.

RFC 4' were cultured at varying cell numbers with LPS (10 μg/ml), and the level of proliferation measured on day 4. Optimal proliferation was obtained with 6 x 10^3 RFC/well in the presence of LPS (Figure 21). At higher cell densities (12 x 10^3 to 24 x 10^3 RFC/well), increased background proliferation in the absence of LPS and reduced proliferation in the presence of LPS was observed.

Proliferation induced by EL-4 sup (1:20 dilution) was dependent on the cell number/well and higher cell densities induced a small but significant increase in thymidine uptake. The stimulation indexes in the presence of EL-4 sup in cultures containing 3 x 10^3, 6 x 10^3, 12 x 10^3, and 24 x 10^3 rosettes/well were 3.2, 4.6, 9.6, and 10, respectively. For reasons discussed earlier, 6 x 10^3 rosettes/well were chosen for further experiments. In the experiments reported here, the increase in thymidine incorporation in the presence of EL-4 sup alone, was always less than 3-fold relative to unstimulated RFC 4' controls.

In the presence of LPS and EL-4 sup, RFC 4' differentiate into IgM-secreting cells (Table 15). This is consistent with the observation described in Table 7, where LPS and EL-4 sup were shown to induce RFC 25' to generate IgM PFC. It suggests that the requirement of signals to induce antigen-specific PFC remains the same for RFC 25' and RFC 4'.

Table 14

^{86}Rb$^+$ Influx in RFC 4'[1] Stimulated with LPS and/or EL-4 Sup

Cell Culture	Experiment[3]		
	1	2	3
RFC	132 ± 0.01	148 ± 0.04	116 ± 0.72
RFC + LPS	210 ± 0.05	206 ± 0.06	223 ± 0.63
RFC + EL-4 Sup	206 ± 8.31	196 ± 0.08	177 ± 0.47

[1] RFC 4' were precultured at 5 x 10^4 cells/ml for 3-4 hr before adding LPS and/or EL-4.

[2] After 14 hr of culture, ^{86}Rb$^+$ uptake was measured at 1 and 21 min of assay as described in *Materials and Methods*. Results are expressed in pmol/10^6 cells/min ± S.D.

[3] ^{86}Rb$^+$ uptake is shown in each column from three representative experiments.

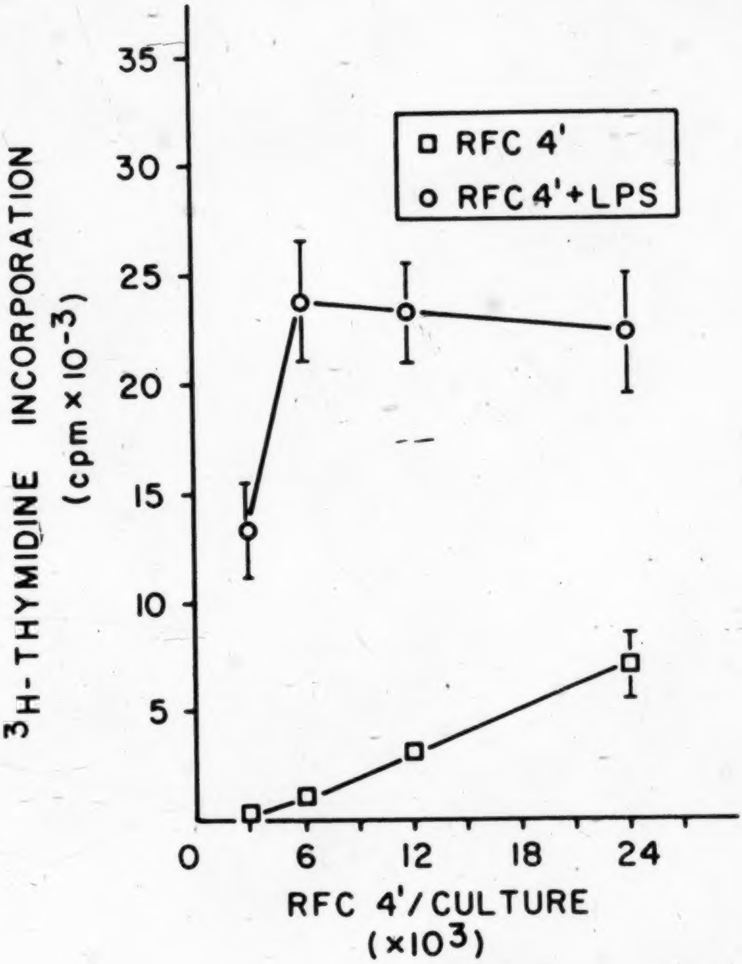

Figure 21. Effect of different concentrations of RFC 4' on activation of proliferation with LPS. Varying numbers of RFC 4' were cultured in the absence and presence of LPS (10 μg/ml). After 4 days, cultures were pulsed with ^{3}H-thymidine for 4 hr and harvested. Data represent means of triplicate cultures expressed as cpm/culture ± S.D.

Table 15

Effect of LPS and EL-4 Sup on Proliferation and Differentiation of RFC 4'[1]

Stimuli	^{3}H-Thymidine Incorporation[2] (cpm/culture) ± S.D.	PFC/culture[3] ± S.E.
None	190 ± 36	<1
LPS	16,632 ± 1,350	56 ± 16
EL-4 Sup	360 ± 76	<1
LPS + EL-4 Sup	ND[4]	500 ± 83

[1] RFC 4' were cultured at 6 x 10^5 cells/well in the presence of 10 μg/ml of LPS or 50 μg/ml of EL-4 sup (1:20 dilution).

[2] Cultures were pulsed at 4 days with ^{3}H-thymidine for 4-6 hr and harvested.

[3] Cultures were assayed on day 4.5 for anti-B^2 IgM PFC. Data represent means of triplicate experiments.

[4] Not done.

IL-2 Induces the PFC Response by LPS-Stimulated RFC 4'

Activated B cells have been shown to express IL-2 receptors and to proliferate in response to r IL-2. Nakanishi *et al* (1984) defined two differentiation-inducing factors in supernatants from PMA-activated EL-4 cells. One of these co-migrates with IL-2. Pike *et al* (1984) demonstrated that human IL-2 can promote both growth and differentiation of single hapten-specific murine B cells in the presence of specific antigen. In the present study, it was, therefore, considered possible to replace the differentiation activity of EL-4 sup with other cytokines such as IL-2. Table 16 demonstrates the effect of human r IL-2 on the differentiation of RFC 4'. RFC 4' stimulated with LPS and r IL-2 did, indeed, generate a PFC response, while IL-2 alone had no effect. This demonstrates that both EL-4 sup and r IL-2 have differentiative activity. The PFC response obtained in the presence of LPS and EL-4 sup was, however, higher than that observed in the presence of LPS and r IL-2.

IL-1 Enhances the IL-2-Induced PFC Response by LPS-Stimulated RFC 4'

It was consistently observed that RFC 25' stimulated with LPS and EL-4 sup generate a higher PFC response than do RFC 4'. One obvious difference between RFC 25' and RFC 4' is the smaller number of nonrosetted cells contaminating the rosettes with the latter preparation. These cells are retained in the interphase during the Ficoll spin. Possibly, these nonrosetted cells represent, at least in part, macrophages that released IL-1. If in the RFC 4' these cells were limiting, reconstitution should be possible with IL-1. Table 17 shows the effect of adding human r IL-1 to RFC 4' stimulated with LPS and EL-4 sup. Clearly, IL-1 significantly enhanced the PFC response. Similarly, the RFC 4' response to LPS and IL-2 underwent a significant enhancement in the presence of IL-1 (Table 18). IL-1 was insufficient to induce a PFC response by RFC 4' stimulated with LPS in the absence of IL-2. Maximal enhancement was observed in the presence of 400 to 200 U/ml of r IL-1. None of the other combinations of stimuli, i.e., LPS and IL-1 or IL-2, or IL-1 and IL-2, induced any PFC response (Table 19). These results demonstrated that r IL-1 is required as an additional differentiation signal for the generation of a PFC response induced by LPS and r IL-2. Table 19 summarizes the results obtained from two representative experiments.

Table 16

Effect of Recombinant IL-2 on Differentiation of RFC 4'

Lymphokine[1]	PFC/culture ± S.E.[2]	
	-LPS	+LPS
None	<1	36 ± 21
EL-4 Sup	<1	4,444 ± 349
r IL-2 (500.0 U/ml)	<1	1,116 ± 159
r IL-2 (250.0 U/ml)	<1	712 ± 150
r IL-2 (50.0 U/ml)	<1	928 ± 31
r IL-2 (5.0 U/ml)	<1	156 ± 22
r IL-2 (0.5 U/ml)	<1	8 ± 5

[1] RFC 4' were cultured at 6 x 10^3 cells/well in the presence of 10 μg/ml of LPS, 50 μl/ml of EL-4 sup (1:20 dilution), or different concentrations of r IL-2.

[2] Cultures were assayed on day 4.5 for anti-B^2 IgM PFC. Data represent means of triplicate experiments.

Table 17

Effect of Recombinant IL-1 on Differentiation of RFC 4' and RFC 25' by LPS and EL-4 Sup

Stimuli[1]	PFC/culture ± S.E.[2]	
	RFC 25'	RFC 4'
None	<1	<1
LPS	<1	<1
EL-4 Sup	<1	<1
LPS + EL-4 Sup	1,272 ± 63	264 ± 23
LPS + r IL-1	<1	<1
LPS + EL-4 Sup + r IL-1	2,020 ± 402	1,808 ± 403

[1] RFC 4' and RFC 25' were cultured at 6 x 10^3 cells/well and 3 x 10^3 cells/well, respectively, in the presence of 10 μg/ml of LPS, 50 μg/ml of EL-4 sup (1:20 dilution), or 200 U/ml of r IL-1.

[2] Cultures were assayed on day 4.5 for anti-B^1 IgM PFC. Data represent means of triplicate experiments.

Table 18

Dose Response of r IL-1-Induced Enhancement of Differentiation

Stimuli[1]	Dose of IL-1 (U/ml)	PFC/culture[2]
None	-	<1
LPS	-	36 ± 21
EL-4 Sup	-	<1
LPS + EL-4 Sup	-	1,052 ± 346
LPS + r IL-2	-	918 ± 188
LPS + r IL-1	800	160 ± 21
LPS + r IL-2 + r IL-1	800	1,662 ± 136
LPS + r IL-2 + r IL-1	400	1,950 ± 328
LPS + r IL-2 + r IL-1	200	2,280 ± 640
LPS + r IL-2 + r IL-1	100	1,306 ± 145
LPS + r IL-2 + r IL-1	50	1,060 ± 104

[1] RFC 4' were cultured at 6 x 10^3 cells/well in the presence of 10 μg/ml of LPS or 500 units/ml of r IL-2.

[2] Cultures were assayed on day 4.5 for anti-B^2 IgM PFC. Data represent means of triplicate experiments ± S.E.

Table 19

Effect of Recombinant IL-2 and IL-1 on Differentiation of RFC 4'

Stimuli[1]	PFC/culture[2]	
	Experiment 1	Experiment 2
None	<1	<1
LPS	70 ± 9	312 ± 122
EL-4 Sup	<1	ND[3]
LPS + EL-4 Sup	1,052 ± 346	ND
r IL-1	<1	<1
r IL-2	<1	<1
r IL-1 + r IL-2	<1	<1
LPS + r IL-1	208 ± 41	320 ± 55
LPS + r IL-2	918 ± 188	1,256 ± 51
LPS + r IL-2 + r IL-1	1,950 ± 328	5,260 ± 372

[1] RFC 4' were cultured at 6 x 10^3 cells/well in the presence of 10 μg/ml of LPS, 50 μl/ml of EL-4 sup (1:20 dilution), 500 units/ml of r IL-2, or 400 units/ml of r IL-1.

[2] Cultures were assayed on day 4.5 for anti-B^2 IgM PFC. Data represent means of triplicate experiments ± S.E.

[3] Not done.

Effect of IL-2 and IL-1 on the PFC Response by RFC 4' Depleted of T Cells and Adherent Cells

RFC 4' depleted of adherent and Thy-1.2⁺ cells prior to rosetting was used in the following study. These RFC 4' contain as little as 0.9 ± 0.5 percent non specific esterase-positive cells and 0.6 ± 0.2 percent Thy-1.2⁺ cells. Such purified RFC 4' were shown to respond to LPS and EL-4 sup by generation of IgM PFC (Table 20). This rules out the possibility that the differentiation of RFC 4' observed in the presence of EL-4 sup was due to an indirect effect from contaminating T cells. Furthermore, RFC 4' depleted of T cells and macrophages, when stimulated with LPS and r IL-2, generated a PFC response that was significantly increased by the additional presence of r IL-1 (Table 21). Data in Tables 21 and 19 indicate that optimal degrees of LPS-stimulated differentiation of B cells into PFC in the presence of r IL-2 requires additional signals delivered from r IL-1.

Decreasing the concentration of r IL-2 diminishes the PFC response, both in the presence of LPS only or LPS and r IL-1 (Table 22). The additional requirement for r IL-1 in the generation of a maximal PFC response by r IL-2 and LPS, however, is seen at all dilutions of r IL-2. Even at a very low concentration of r IL-2 (5 U/ml), IL-1 promoted significant enhancement of the PFC response.

The effect of r IL-2 and r IL-1 on LPS-induced proliferation of purified RFC 4' is shown in Table 23. IL-1 and r IL-2, alone or in combination, at a concentration found to be optimal for induction of a PFC response did not induce proliferation. IL-2 consistently enhanced the proliferation of RFC in the presence of LPS. The enhancement of the proliferative response in the presence of IL-1 varied from being significant to marginal. No additive effect on proliferation was observed on adding IL-1 and IL-2 to RFC in the presence of LPS .

IL-2 and IL-1 are Both Required During the Early Phase of the B Cell Response to LPS

The PFC response kinetics by RFC 4' depleted of T cells and adherent cells upon addition at different points in time of LPS, r IL-2, and r IL-1, are shown in Figure 22. RFC did not respond to stimulation by r IL-2 when the addition of LPS was delayed for 24 hrs

Table 20

Effect of LPS and EL-4 Sup on Proliferation and Differentiation of RFC 4' Depleted of Adherent and Thy-1.2-Positive Cells

Stimuli[1]	^{3}H-Thymidine Incorporation[2] (cpm/culture) ± S.D.	PFC/culture[3]
None	366 ± 75	<1
LPS	34,214 ± 368	176 ± 48
EL-4 Sup	242 ± 34	<1
LPS + EL-4 Sup	ND[4]	1,916 ± 168

[1] RFC 4' depleted of adherent and Thy-1.2-positive cells before rosetting were cultured at 6 x 10^5 cells/well in the presence of 10 μg/ml of LPS or 50 μl/ml of EL-4 sup (1:20 dilution).

[2] Cultures were pulsed at 4 days with ^{3}H-thymidine for 4-6 hr and harvested.

[3] Cultures were assayed on 4.5 days for anti-*B*[2] IgM PFC. Data represent means of triplicate experiments ± S.E.

[4] Not done.

Table 21

Effect of Recombinant IL-2 and IL-1 on Differentiation of RFC 4'
Depleted of Adherent and Thy-1.2-Positive Cells

Stimuli[1]	PFC/culture[2]	
	Experiment 1	Experiment 2
None	<1	<1
LPS	34 ± 18	20 ± 14
EL-4 Sup	<1	ND[3]
LPS + EL-4 Sup	572 ± 40	ND
r IL-1	<1	<1
r IL-2 (500)	<1	<1
r IL-2 (50)	<1	<1
r IL-2 + r IL-1	<1	<1
LPS + r IL-1	52 ± 23	40 ± 14
LPS + r IL-2 (500)	312 ± 81	292 ± 95
LPS + r IL-2 (50)	280 ± 59	316 ± 90
LPS + r IL-2 (500) + r IL-1	1,058 ± 207	1,744 ± 349
LPS + r IL-2 (50) + r IL-1	436 ± 48	1,040 ± 259

[1] RFC 4' depleted of adherent and Thy-1.2-positive cells before rosetting were cultured at 6 x 10^3 cells/well in the presence of 10 μg/ml of LPS, 50 and 500 units/ml of r IL-2, or 400 units/ml of r IL-1.

[2] Cultures were assayed on 4.5 days for anti-B^2 IgM PFC. Data represent means of triplicate experiments ± S.E.

[3] Not done.

Table 22

Dose Response Effect of r IL-2 and r IL-1 on Differentiation of LPS-Activated Cells

r IL-1 Concentration (U/ml)	r IL-2 Concentration (U/ml)				
	500.0	50.0	5.0	0.5	None
400.0	1,384 ± 189	924 ± 162	228 ± 39	76 ± 22	76 ± 23
50.0	724 ± 189	720 ± 48	228 ± 11	68 ± 3	68 ± 23
5.0	440 ± 213	536 ± 83	68 ± 7	84 ± 20	40 ± 11
0.5	144 ± 46	224 ± 17	12 ± 7	24 ± 7	12 ± 9
None	132 ± 9	92 ± 59	32 ± 7	12 ± 7	20 ± 11

RFC 4' depleted of adherent and Thy-1.2-positive cells were cultured at 6 x 10^3 cells/well in the presence of 10 μg/ml of LPS. Recombinant IL-2 and IL-1 were added at different concentrations to cell cultures. The cultures were assayed on day 4.5 for anti-B^2 IgM PFC. Data represent means of triplicate experiments and are expressed as PFC/culture ± S.E.

Table 23

Effect of Recombinant IL-2 and IL-1 on Proliferation of RFC 4' Depleted of Adherent and Thy-1.2-Positive Cells

Stimuli[1]	^{3}H-Thymidine Incorporation[2] (cpm/culture) ± S.D.	
	Experiment 1	Experiment 2
None	229 ± 36	281 ± 80
LPS	13,959 ± 765	15,329 ± 1,878
r IL-1	409 ± 192	293 ± 36
r IL-2	378 ± 82	255 ± 53
r IL-1 + r IL-2	369 ± 122	306 ± 66
LPS + r IL-1	23,337 ± 1,291	17,764 ± 1,289
LPS + r IL-2	28,052 ± 1,051	22,618 ± 767
LPS + r IL-2 + r IL-1	29,054 ± 1,416	23,724 ± 2,796

[1] RFC 4' depleted of adherent and Thy-1.2-positive cells before rosetting were cultured at 6 x 10^3 cells/well in the presence of 10 μg/ml of LPS, 500 units/ml of r IL-2, or 400 units/ml of r IL-1.

[2] Cultures were pulsed on day 4 with ^{3}H-thymidine for 4-6 hr and harvested. Data represent means of triplicate experiments.

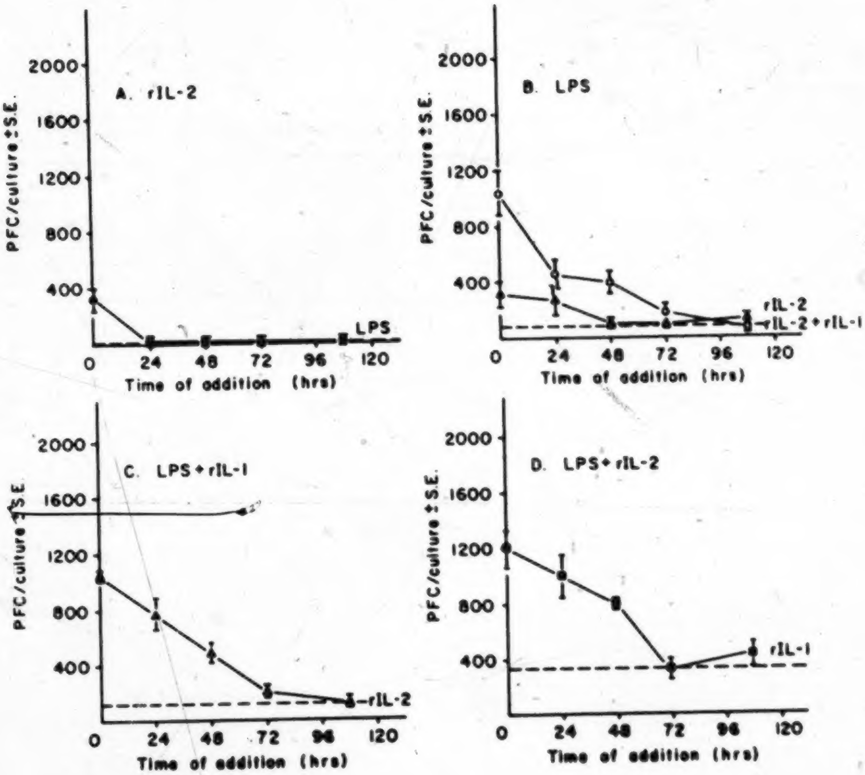

Figure 22. Kinetics of induction of PFC by addition of LPS, r IL-2, or r IL-1. RFC 4' depleted of adherent and Thy-1.2-positive cells were cultured at 6 x 10^5 cells/well with (A) r IL-2 (500 U/ml), (B) LPS (10 μg/ml), (C) LPS and r IL-1 (400 U/ml), or (D) LPS and r IL-2 (500 U/ml). LPS (A), r IL-2 or r IL-2 plus r IL-1 (B), r IL-2 (C), or r IL-1 (D) were added to the cultures at different time intervals. Cultures were assayed at 112 hr for anti-B^2 IgM PFC. Data represent means of triplicate experiments. The dotted line indicates PFC per control culture.

after the onset of culture (A). The addition of r IL-2 or r IL-2 plus IL-1 to RFC cultures stimulated with LPS could be delayed upto 24 hrs without significantly decreasing the PFC response (B). This is consistent with the conclusion that the ability of r IL-2 to induce differentiation is dependent on prior proliferative signals provided by LPS. A maximal enhancement of the PFC response was observed when r IL-2 and IL-1 were both added at the onset of culture to RFC stimulated with LPS (C,D). Significant enhancement of the PFC response could be seen even when the addition of IL-1 to LPS plus IL-2 stimulated RFC, and of IL-2 to LPS plus IL-1 stimulated RFC was delayed upto 48 hrs after the onset of culture (C,D). Finally, we were unable to find any difference in stimulatory activity between human and mouse r IL-1 (Table 24).

Discussion

In order to understand the B cell response, it is critical to evaluate the role of different lymphokines on B cell maturation. Our method of B cell purification provides pure and unactivated B cells. Preparation of RFC that contained very few contaminating non-RFC were obtained by 4 min Ficoll-centrifugation. Ninety-nine percent of rosettes from the RFC 4' preparation were IgM$^+$. At the functional level, RFC, RFC 4'and purified RFC 4' required the same set of signals, LPS and EL-4 sup, for proliferation and differentiation , in spite of the differences between the two preparations in the numbers of nonrosetted cells (Tables 7, 21 and 15). RFC 4' that had been depleted of Thy-1.2$^+$ cells and adherent cells were found to respond to LPS and EL-4 sup or LPS and r IL-2 in the same manner as RFC 4' that had not been additionally purified (Tables 19 and 21). This rules out the possibility that r IL-2 had an indirect effect on rosetted B cells. However, LPS together with r IL-2 was less effective in inducing PFC from purified RFC 4' than LPS and EL-4 sup. The discrepancy we observed in the number of PFC generated upon stimulation of RFC 4' with LPS and EL-4 sup when compared to LPS and r IL-2, was probably due to differences in the differentiation inducing ability of IL-2 and EL-4 sup. From the previous section (p. 66), it is clear that the ability of EL-4 sup to induce differentiation is not due to IL-2 contained in the preparation.

Table 24

Effect of Recombinant Mouse and Human IL-1 on Differentiation

Stimuli[1]	PFC/culture ± S.E.[2]
None	<1
LPS	176 ± 48
LPS + r mIL-1	156 ± 21
LPS + r IL-2	584 ± 37
LPS + r IL-2 + r mIL-1	1,240 ± 97
LPS + r hIL-1	200 ± 34
LPS + r IL-2 + r hIL-1	1,224 ± 222

[1] RFC 4' depleted of adherent and Thy-1.2-positive cells were cultured at 6 x 10^5 cells/well in the presence of 10 μg/ml of LPS, 500 U/ml of r IL-2, or 400 U/ml of r mIL-1 or r hIL-1.

[2] Cultures were assayed on day 4.5 for anti-*B*² IgM PFC. Data represent means of triplicate experiments.

Contradictory statements concerning the requirement for cytokines in B cell activation have been reported. In this section, this question was addressed using purified RFC 4' that could be cultured at low cell density (6 x 10^3/0.2 ml culture) to yield an antibody response when appropriately stimulated. In such cultures, T cells and macrophages were functionally limiting. Thus, purified RFC 4' preparations contained on the average as few as 0.6 ± 0.2% Thy-1.2$^+$ cells and 0.9 ± 0.5% monocytes that were positive for nonspecific esterase. The number of contaminating cells per RFC culture is approximately 36-48 Thy-1.2$^+$ cells and 54-98 nonspecific esterase-positive cells. These numbers are not significant enough to influence the PFC response. In fact, addition of up to 12 x 10^4 cells, obtained from the interphase during the last Ficoll spin (mainly T cells and monocytes), does not induce a PFC response from RFC 4' stimulated with IL-2 or LPS. This argues against the ability of IL-2 to stimulate the small numbers of contaminating non-B cells to release B cell stimulatory factors in these cultures. However, the PFC response obtained in the presence of LPS and IL-2 is enhanced by the addition of 6-12 x 10^4 cells to RFC 4' cultures (data not shown).

In this section, it was demonstrated that r IL-2 is sufficient to induce B cells to differentiate into antibody-forming cells in the presence of LPS (Table 21). Nakanishi *et al* (1984) have also reported a role of r IL-2 in B cell differentiation of anti-IgM-activated B cells, except that BSF-1 (IL-4) and B151-TRF (IL-5) were also required. Although IL-2 was sufficient to trigger proliferating B cells to differentiate, r IL-1 was shown to enhance the effect of r IL-2 (Table 21). In contrast to reports by Pike *et al* (1984), we were unable to confirm that IL-2 is able to induce resting B cells to differentiate in the absence of LPS.

An IgM response induced by T-independent antigens (TI-1 and TI-2), including polyclonal activators, depends on the presence of macrophages (reviewed by Corbel and Melchers, 1983). In this study, r IL-1 was shown not to induce differentiation of B cells either in the presence or absence of LPS (Table 21). This again differs from the reports by Pike and Nossal (1985), who showed that IL-1 alone could induce differentiation of Flu-Pol-stimulated B cells. Although the reason for this difference could be the single-cell culture technique used by these authors, it is interesting that Pol is also a polyclonal B cell activator (Scott and Diener, 1976).

There was a dramatic increase of 400-800 percent in the number of PFC generated in the presence of both r IL-1 and r IL-2 (Tables 19 and 21). This is in contrast to the 50-100 percent enhancement of the antibody response reported by others in the presence of IL-1 using both murine and human B cells (Booth and Watson, 1984; Liebson *et al*, 1981; Falkoff *et al*, 1984; Fugui and Almquist, 1986). Shirakawa *et al* (1986) have reported that IL-1 induces B cells to generate IL-2 receptors. It is possible that the synergism between IL-1 and IL-2 reflects a more effective IL-2 binding by B cells due to an increase in IL-2 receptor density in response to IL-1.

It has been reported that resting B cells express few if any receptors for IL-2 (Zubler *et al*, 1984; Robb *et al*, 1984; Lowenthal *et al*, 1985). Velocity sedimentation studies reveal that 99 percent of the rosettes in purified RFC 4' preparations were formed by small-sized IgM$^+$ B cells (data not shown). Hence, the conclusion that purified RFC 4' probably do not express IL-2 receptors before stimulation with LPS.

Hashimoto *et al* (1986) reported that responsiveness of B cells to IL-2 is evident in the presence of LPS and a surface Ig-mediated signal which in turn induces IL-2 receptors. B cells stimulated with LPS alone were not responsive to IL-2. RFC used in these experiments depend on the binding of CRBC by surface Ig receptors (Shiozawa *et al*, 1980). Therefore, purified RFC 4' in the presence of LPS are probably in a functionally similar state of activation as are B cells stimulated with anti-IgM and LPS (Hashimoto *et al*, 1986).

The 500 units/ml of r IL-2 required to stimulate B cells in the system reported here or 500-1000 units/ml reported by others (Nakanishi *et al*, 1984) may appear excessive. Conceivably, however, lymphokines may occur at such concentrations in the microenvironment of secondary lymphoid organs.

It was earlier shown that IL-1α from human and mouse could be used to stimulate purified RFC 4' (Table 24). The two members of the IL-1 family, IL-1α and IL-1β, show limited homology between the protein sequences but share many biological properties (reviewed by Oppenheim *et al*, 1986). IL-1α and IL-1β also bind to identical receptors on murine and human B cells (Dower *et al*, 1986). It remains to be determined whether IL-1α and IL-1β have identical biological activities as B cells.

IV. Summary

B cell activation can be studied using either tumor model systems or by analyzing polyclonal activation of splenic B cells. The research presented in this thesis was undertaken to examine the requirements to induce proliferation and differentiation of antigen affinity-enriched B cells and to link at least some of the early biochemical changes and their functional consequences for B cell proliferation and maturation.

B cells specific for the B^2 haplotype of CRBC were affinity-enriched by rosetting spleen cells with CRBC of the B^2 haplotype. This method selects a highly enriched population of antigen-binding B cells (>99 percent IgM$^+$ B cells) that can be cultured at 3-6x10^3 RFC/well. These B cells (RFC) were used to design a model system of B cell activation. Addition of LPS, a polyclonal activator, triggered B cells to proliferate, without maturing into AFC. For differentiation to occur, B cells required both LPS and a source of T cell lymphokines. In this case, PMA-induced EL-4 sup was used as a source of T cell lymphokines. This suggested that LPS provides the stimulus for a proliferative signal while the EL-4 sup induces maturation to PFC. Both LPS and EL-4 sup are required early (day 1) in the PFC response by RFC.

Having thus defined our basic B cell activation model, we went on to look at a panel of biochemical changes suggested to occur early in cell activation, in this system. The aim was to search for both negative and positive correlations between B cell activation and these early biochemical changes. The panel of parameters were analyzed in unactivated RFC, and RFC activated with LPS alone, EL-4 sup alone, and LPS + EL-4 sup. Two major conclusions appeared from these studies. First, the activation of Na$^+$/K$^+$ ATPase was triggered by LPS as well as by EL-4 sup alone, although EL-4 sup alone, unlike LPS, did not induce an increase in the transport of proline, RNA, protein, and DNA synthesis. We must thus conclude that activation of Na$^+$/K$^+$ ATPase is in itself not sufficient to cause B cells to proliferate. Secondly, LPS induces an increase in cytoplasmic μ mRNA and IgM secretion but does not induce PFC. When LPS plus EL-4 sup were used to induce RFC, there was a consistent increase in cytoplasmic μ mRNA and IgM secretion.

We decided to investigate if certain recombinant cytokines could replace EL-4 sup in our model system. Spleen cells were depleted of adherent and Thy-1.2$^+$ cells before rosetting to rule out any indirect effects caused by these cytokines. These preparation of RFC 4' contain as little as 0.6 ± 0.2 percent of Thy-1.2$^+$ and 0.9 ± 0.5 percent nonspecific esterase-positive cells. Recombinant IL-2, like EL-4, had no effect on the proliferation of RFC in the absence of LPS. However, in the presence of LPS and IL-2, RFC 4' are stimulated to mature into PFC. This PFC response obtained in the presence of LPS and r IL-2 could be enhanced several fold by r IL-1. For optimal effects, both r IL-1 and r IL-2 were required early in the response. These studies have demonstrated that IL-1 and IL-2 induce the differentiation to PFC of LPS-stimulated B cells. Both were required for optimal differentiation.

In summary, a model polyclonal for B cell activation is described and the lymphokines required for B cell differentiation have been defined.

VII. References

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